Dexamethasone-induced and estradiol-induced CREB activation and annexin 1 expression in CCRF-CEM lymphoblastic cells: evidence for the involvement of cAMP and p38 MAPK

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Aims: Annexin 1 (ANXA1), a member of the annexin family of calcium-binding and phospholipid-binding proteins, is a key mediator of the anti-inflammatory actions of steroid hormones. We have previously demonstrated that, in the human lymphoblastic CCRF-CEM cell line, both the synthetic glucocorticoid hormone, dexamethasone (Dex), and the estrogen hormone, 17β-estradiol ($E_2β$), induce the synthesis of ANXA1, by a mechanism independent of the activation of their nuclear receptors. Recently, it was reported that the gene coding for ANXA1 contains a cAMP-responsive element (CRE). In this work, we investigated whether Dex and $E_2β$ were able to induce the activation of CRE binding proteins (CREB) in the CCRF-CEM cells. Moreover, we studied the intracellular signalling pathways involved in CREB activation and ANXA1 synthesis in response to Dex and $E_2β$; namely, the role of cAMP and the p38 mitogen-activated protein kinase (MAPK).

Results: The results show that Dex and $E_2β$ were as effective as the cAMP analogue, dBcAMP, in inducing CREB activation. On the contravy, dBcAMP induced ANXA1 synthesis as effectively as these steroid hormones. Furthermore, the cAMP antagonist, Rp-8-Br-cAMPS, and the specific p38 MAPK inhibitor, SB203580, effectively prevented both Dex-induced, $E_2β$-induced and dBcAMP-induced CREB activation and ANXA1 synthesis.

Conclusions: Taken together, our results suggest that, in CCRF-CEM cells, Dex-induced and $E_2β$-induced ANXA1 expression requires the activation of the transcription factor CREB, which in turn seems to be mediated by cAMP and the p38 MAPK. These findings also suggest that, besides the nuclear steroid hormone receptors, other transcription factors, namely CREB, may play important roles in mediating the anti-inflammatory actions of glucocorticoids and oestrogen hormones.

Key words: Annexin 1, CREB, 17β-Estradiol, Dexamethasone, cAMP, p38 MAPK

Introduction

Annexin 1 (ANXA1) (also known as lipocortin 1) is a member of the annexin family of calcium-binding and phospholipid-binding proteins,[1,2] which is constitutively expressed in many human cells, particularly in polymorphonuclear leukocytes.[1,3] Recently, we observed that ANXA1 is also constitutively expressed in a human lymphoblastic T cell line, the CCRF-CEM cell line.[4] Although its biological role is still incompletely characterised, ANXA1 is considered an endogenous anti-inflammatory effector that mediates at least some of the anti-inflammatory actions of the glucorticoid (GC) hormones; namely, the inhibition of phospholipase A_2, with the consequent suppression of prostaglandin and leukotriene production,[1] the inhibition of superoxide generation,[5]

the inhibition of the activation, transmigration and phagocytic ability of neutrophils.[3] Recently, ANXA1 has also been shown to mediate the inhibitory effect of GCs on the expression of cyclooxygenase-2 (COX-2),[6–8] an enzyme required for prostaglandin production and also involved in cell proliferation.[9,10] Furthermore, some of the anti-inflammatory effects attributed to 17β-estradiol ($E_2β$), the most potent oestrogen hormone, are identical to those exerted by ANXA1, suggesting that this protein may also mediate the anti-inflammatory actions of $E_2β$.[11,12]

In resting cells ANXA1 is an intracellular protein, being secreted and resynthesised upon stimulation. Once in the extracellular medium, ANXA1 binds, in a paracrine or autocrine way, to specific plasma membrane binding sites, which are considered to mediate its biological actions.[5,13] Consistent with the

notion that ANXA1 mediates some of the anti-inflammatory actions of GCs, these hormones, most remarkably the synthetic GC dexamethasone (Dex), have been shown to potently induce ANXA1 secretion and synthesis in many different cells.[14,15] Our own observations also show that both Dex[16] and $E_2\beta$[4] induce the rapid secretion of ANXA1, followed by its *de novo* synthesis, in the human lymphoblastic CCRF-CEM cells.

The intracellular signalling pathways utilised by either the GC or the oestrogen hormones to induce ANXA1 synthesis are still poorly understood. Many actions of these hormones are mediated through genomic mechanisms involving their respective nuclear receptors, which, upon binding the hormone, act as transcription factors directly modulating the transcription of genes containing in their promoter regions either a GC responsive element (GRE) or an oestrogen responsive element.[17,18] Using the potent GC receptor antagonist RU486 (mifepristone), some studies showed that, in some cells, the synthesis of ANXA1 is dependent on GC receptors,[2,19] whereas the opposite was found in other cells.[20,21] Therefore, some authors have suggested that the putative GRE that is present in the promoter region (intron I) of the ANXA1 gene is non-functional.[22,23] In our previous work, we also found no evidence supporting the involvement of such nuclear hormone receptors in either Dex-induced or $E_2\beta$-induced ANXA1 synthesis in CCRF-CEM cells.[4,16]

Besides the putative GRE, it was recently shown that the promoter region of the ANXA1 gene contains a cAMP-responsive element (CRE) and that a CRE-binding protein (CREB) is required for either Dex-induced or cAMP-induced ANXA1 synthesis.[22] Furthermore, Dex and $E_2\beta$ have also been shown to induce the transcription of several genes by activating CREB.[22,24,25]

The phosphorylation and consequent activation of CREB can be regulated by several intracellular signalling pathways. Among those, cAMP[26,27] and the p38 mitogen-activated protein kinase (MAPK)[28,29] seem to play crucial roles in the activation of CREB in different cells. Interestingly, various studies showed that Dex and $E_2\beta$ can stimulate adenylate cyclase activity, thus increasing the production of cAMP,[30−33] which is also an inducer of ANXA1 synthesis in some cell types.[22,34,35] Moreover, recent reports indicate that, in different cells, Dex and $E_2\beta$ can up-regulate the activity of the p38 MAPK.[36−38]

In an attempt to understand the mechanisms by which Dex and $E_2\beta$ induce the synthesis of ANXA1, we have investigated the ability of these two steroid hormones to activate CREB in the CCRF-CEM cells. Moreover, to further elucidate the signalling pathways involved, we also studied the role of cAMP and of the p38 MAPK on Dex-induced and $E_2\beta$-induced CREB activation and ANXA1 expression.

Materials and methods

Chemicals

The human CCRF-CEM acute T lymphoblastic leukaemia cell line was purchased from American Type Culture Collection (Rockville, MD, USA). Foetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). Dibutyryl-cAMP (dBcAMP) was from BIOMOL (USA). Adenosine 3′,5′-cyclic monophosphorothioate, 8-bromo, Rp-isomer, sodium salt (Rp-8-Br-cAMPS) and SB203580 were purchased from Calbiochem (San Diego, CA, USA). The protease inhibitor cocktail was obtained from Roche (Manheim, Germany). The oligonucleotide probes were from Santa Cruz (Santa Cruz, CA, USA). [γ-^{32}P] adenosine triphosphate, T4 polynucleotide kinase and poly (dI-dC)·(dI-dC) were obtained from Pharmacia Biosciences (Piscataway, NJ, USA). The mouse monoclonal antibody against ANXA1 (Clone Lipo 105) was purchased from BabCO (CA, USA). The FITC-conjugated rabbit anti-mouse antibody and the normal rabbit serum were obtained from DAKO (Copenhagen, Denmark). All other reagents were from Sigma Chemical Co. (St Louis, MO, USA).

Culture conditions

The human CCRF-CEM acute T lymphoblastic leukaemia cell line was maintained in RPMI medium supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin and 100 U/ml penicilin, at 37°C, in an atmosphere of 5% CO_2/95% air. Prior to any treatments, CCRF-CEM cells were plated at 5×10^6 cells/well, in six-well culture plates, in RPMI supplemented with antibiotics and 0.5% FCS, for at least 5 h, and maintained in these conditions thereafter. In each experiment, controls consisting of cells incubated in the corresponding volume of culture medium plus the appropriate vehicle were always included.

Cell viability

The viability of CCRF-CEM cells, under the various experimental conditions, was always assessed by the trypan blue exclusion method and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Assessment of MTT reduction by metabolically active cells was made by a colorimetric assay, as previously described.[39]

Preparation of cytoplasmic and nuclear extracts

After washing with ice-cold phosphate-buffered saline (PBS), the cells were lysed in 'buffer 1' (10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 10 mM Tris−HCl, pH 7.5, and the protease inhibitor

cocktail) and incubated on ice, for 15 min. After brief vortexing, the lysates were centrifuged at $2300 \times g$, for 10 min, at $4°C$. The supernatants, cytoplasmic extracts, were collected and stored at $-70°C$ until further use. The pellets obtained were resuspended in 'buffer 2' (300 mM NaCl, 3 mM $MgCl_2$, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethyl-sulfonylfluoride, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 20 mM HEPES buffer, pH 7.5, and the protease inhibitor cocktail), incubated on ice for 45 min and centrifuged at $12,000 \times g$, for 20 min, at $4°C$. The supernatants, nuclear extracts, were also collected and stored at $-70°C$ until further use. The protein concentration of the extracts was determined using the bicinchoninic acid/copper (II) sulphate protein assay kit.

Electrophoretic mobility shift assay

The oligonucleotide probes containing either the consensus binding sequence corresponding to the CRE (5′-AGA GAT TGC CTG <u>ACG</u> TCA GAG AGC TAG-3′) or a mutated inactive form of this oligonucleotide (5′-AGA GAT TGC CTG <u>TGG</u> TCA GAG AGC TAG-3′) were end-labelled with [γ-^{32}P] adenosine triphosphate, by the T4 polynucleotide kinase, and purified through Sephadex G-50 spin columns. Nuclear extracts (12.5 μg protein) were incubated, for 40 min, at $4°C$, with 100,000 cpm of the [γ-^{32}P]-labelled oligonucleotide, in a buffer containing 100 μ/ml poly(dI-dC)·poly(dI-dC), 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 4% (v/v) Ficoll 400, 0.5 mM dithiothreitol, 50 mM KCl, and 1 mg/ml barine serum albumin. The DNA−protein complexes were resolved by electrophoresis on 7% non-denaturating polyacry-lamide gels, in a buffer system containing 0.044 M Tris−Base (pH 8.0), 4.45 mM boric acid and 1 mM EDTA, at constant voltage of 150 V, for 3 h, at room temperature. The gels were then dried and subjected to autoradiography. In competition experiments, a 100-fold excess of unlabelled oligonucleotide was added to the nuclear extracts immediately before the addition of the radiolabelled probe.

Immunocytochemistry assay

After washing in ice-cold PBS, the cells were spread on poly-D-lysine-coated glass slides and air-dried for 30 min. The cells were then fixed (4% (w/v) paraformaldehyde and 2% (w/v) sucrose in PBS), for 15 min, and permeabilised (1% Triton-XlOO in PBS), for 5 min. Background blocking was performed with normal rabbit serum, and detection of total ANXA1 was performed by incubation with a mouse monoclonal anti-ANXA1 antibody (4 μg/ml). After extensive rinsing in PBS, the cells were incubated with a FTTC-conjugated rabbit anti-mouse antibody. We have previously confirmed the specificity of this anti-ANXA1 antibody by western blot analysis.[4,16] Serum and antibodies were diluted in PBS with 0.5% (w/v) bovine serum albumin and 0.2% (v/v) Tween-20, and all the incubations were performed at room temperature, in a humidified chamber. Control experiments for non-specific binding were performed in parallel by omission of the primary antibody.

Results

Dex, E$_2$β and dBcAMP induce CREB activation

To evaluate CREB activation we used electrophoretic mobility shift assay (EMSA) analysis to detect the presence of active CREB proteins, capable of forming complexes with a [γ-^{32}P]-labelled oligonucleotide probe containing the specific consensus binding sequence for this transcription factor. The oligonucleotide used corresponds to the CRE present in the promoter region of target genes.

CCRF-CEM cells were cultured in RPMI medium supplemented with 0.5% FCS, and treated with Dex (1 μM), E$_2$β (1 μM) or with the stable cell-permeable cAMP analogue dBcAMP (1 mM) for 15 min, 30 min or 1 h. The respective nuclear extracts were used for EMSA analysis. The results obtained show the presence of retarded bands, the intensity of which was much higher in the nuclear extracts from cells treated with Dex (Fig. 1), E$_2$β (Fig. 2) or dBcAMP (Fig. 3) than in those from control untreated cells (Figs. 1−3, lane 1). For each stimulus, the intensity of those bands varied with the incubation time and was maximal when the cells were treated with Dex for 1 h (Fig. 1, lane 4), E$_2$β for 30 min (Fig. 2, lane 3), or dBcAMP for 15 min (Fig. 3, lane 2). Therefore, for each one of these three stimuli, the respective optimal incubation time was used in all subsequent experiments.

The specificity of the complexes formed was evaluated by incubating the nuclear extracts from Dex-treated, E$_2$β-treated or dBcAMP-treated cells with a [^{32}P]-labelled mutant CREB oligonucleotide probe that differs in two bases from the native CREB oligonucleotide. As shown in lane 5 of Figs. 1−3, no specific CREB complexes could be detected under these experimental conditions. Furthermore, competition assays performed by incubation of the nuclear extracts with the [^{32}P]-labelled CREB oligonucleotide probe and with a 100-fold excess of unlabelled oligonucleotide probes, specific for either CREB, mutant CREB or an unrelated transcription factor, octamer (Oct-1), showed that only the unlabelled CREB probe was effective in preventing the formation of specific complexes between the proteins present in the nuclear extracts and the labelled CREB probe (Figs l−3, lanes 6−8). These results indicate that the retarded bands detected in lanes 1−4
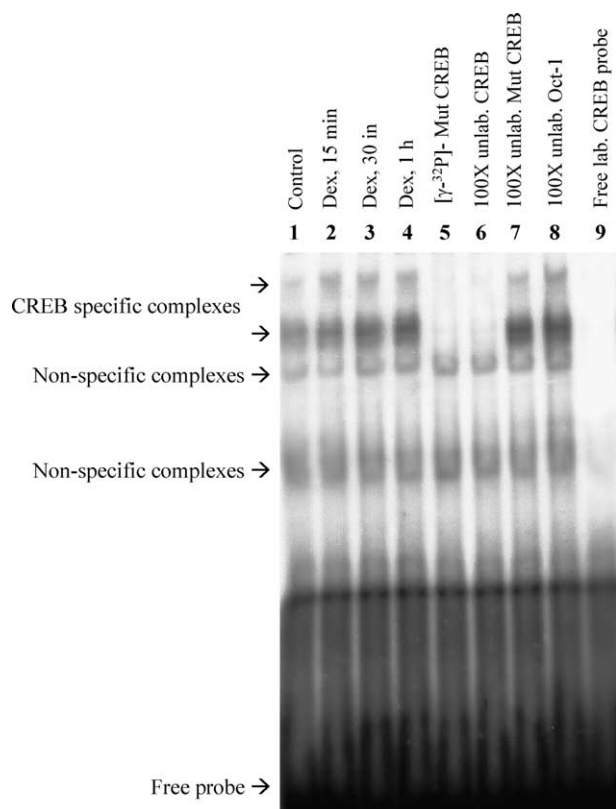
FIG. 1. Dex induces CREB activation in CCRF-CEM cells. The cells were treated with Dex (1 μM) (lanes 2–4), for 15 min, 30 min or 1 h, or left untreated (control, lane 1). Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. Competition experiments with a 100-fold excess of unlabelled competitor probe relative to the labelled CREB probe were performed using the nuclear extracts from cells treated with Dex, for l h (lanes 6–8). EMSA analysis was also performed using a labelled mutant CREB probe (lane 5). The results shown are representative of three independent experiments.

FIG. 2. $E_2\beta$ induces CREB activation in CCRF-CEM cells. The cells were treated with $E_2\beta$ (1 μM) (lanes 2–4), for 15 min, 30 min or 1 h, or left untreated (control, lane 1). Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. Competition experiments with a 100-fold excess of unlabelled competitor probe relatively to the labelled CREB probe were C performed using the nuclear extracts from cells treated with $E_2\beta$, for 30 min (lanes 6–8). EMSA analysis was also performed using a labelled mutant CREB probe (lane 5). The results shown are representative of three independent experiments.

of the autoradiographies presented in Figs. 1–3 represent the formation of specific complexes between CREB proteins and the CREB-specific oligonucleotide probe.

CREB activation, induced by Dex, $E_2\beta$ or dBcAMP, is inhibited by the cAMP antagonist, Rp-8-Br-cAMPS

In order to investigate whether cAMP is required for the activation of CREB induced by Dex, $E_2\beta$ or dBcAMP, we used the potent cAMP antagonist Rp-8-Br-cAMPS, which is metabolically stable and cell permeable.[40] CCRF-CEM cells were pre-treated with Rp-8-Br-cAMPS (500 μM), for l h, before the addition of Dex, $E_2\beta$ or dBcAMP, and then further incubated for l h, 30 min or 15 min, respectively. Assessment of the cell viability by the MTT reduction assay showed that Rp-8-Br-cAMPS did not reduce the viability of the CCRF-CEM cells relatively to untreated cells (data not shown), thus indicating the absence of toxic effects in the experimental conditions used. The results pre-
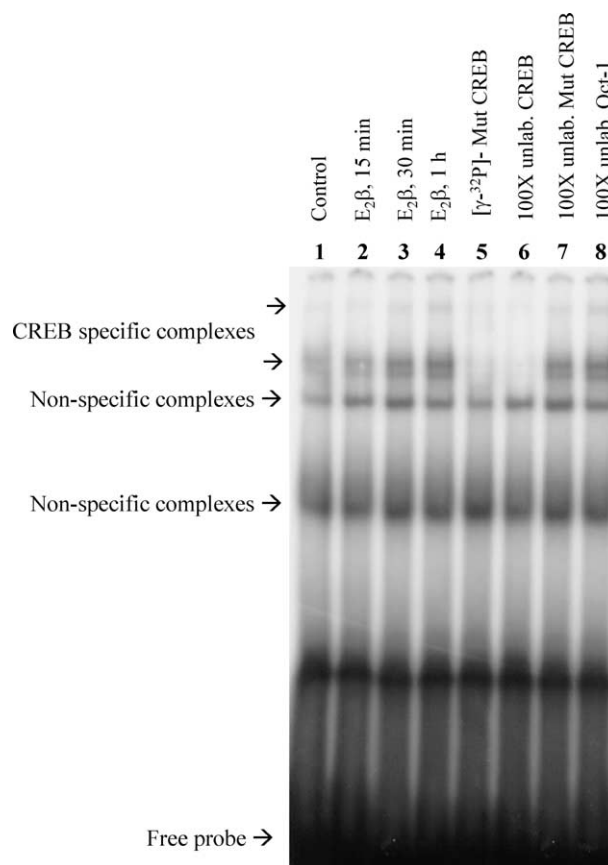
sented in Fig. 4 show that pre-treatment of the cells with Rp-8-Br-cAMPS effectively prevented Dex-induced, $E_2\beta$-induced and dBcAMP-induced CREB activation, as indicated by the reduced intensity of the bands corresponding to the specific CREB complexes (lanes 3, 5 and 7). Treatment of the cells with Rp-8-Br-cAMPS alone had no effect on the basal activation of CREB observed in nuclear extracts from control cells (data not shown).

ANXA1 expression, induced by either Dex, $E_2\beta$ or dBcAMP, is inhibited by the cAMP antagonist Rp-8-Br-cAMPS

ANXA1 expression was evaluated by immunocytochemistry in permeabilised CCRF-CEM cells. This method allowed us to detect total ANXA1, which corresponds to the intracellular protein plus the protein bound to its specific binding sites located on the outer surface of CCRF-CEM cells plasma membrane.
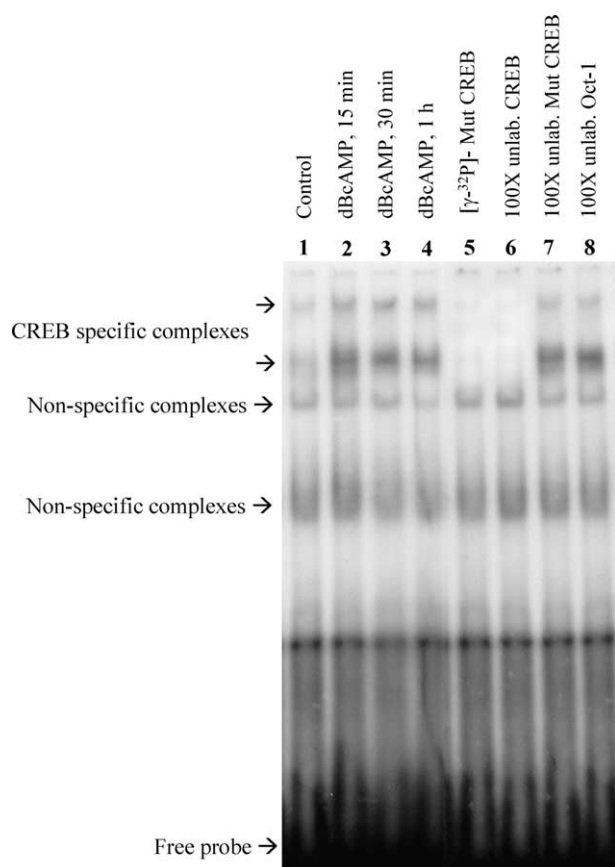
FIG. 3. dBcAMP induces CREB activation in CCRF-CEM cells. The cells were treated with dBcAMP (1 mM) (lanes 2–4), for 15 min, 30 min or 1 h, or left untreated (control, lane 1). Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. Competition experiments with a 100-fold excess of unlabelled competitor probe relatively to the labelled CREB probe were performed using the nuclear extracts from cells treated with dBcAMP, for 15 min (lanes 6–8). EMSA analysis was also performed using a labelled mutant CREB probe (lane 5). The results shown are representative of three independent experiments.
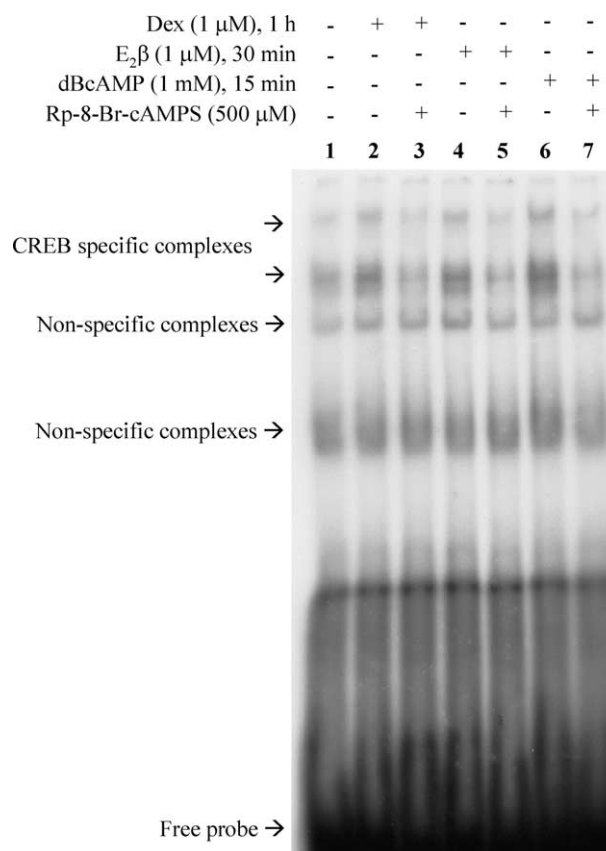

FIG. 4. Rp-8-Br-cAMPS inhibits Dex-induced, $E_2\beta$-induced and dBcAMP-induced CREB activation in CCRF-CEM cells. The cells were incubated in the presence or in the absence (control, lane 1) of the cAMP antagonist Rp-8-Br-cAMPS (500 μM), for 1 h. Then, the cells were further incubated with Dex (1 μM), $E_2\beta$ (1 μM) or dBcAMP (1 mM) for 1 h, 30 min or 15 min, respectively. Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. The results shown are representative of three independent experiments.

We have previously shown that Dex significantly increases the cellular content of ANXA1, upon stimulation of CCRF-CEM cells for 12 h.[16] Therefore, in this work the cells were treated with Dex (1 μM), $E_2\beta$ (1 μM) or dBcAMP (1 mM), for 12 h, for the detection of total ANXA1 by immunocytochemistry.

The results obtained showed that control untreated CCRF-CEM cells have a weak basal fluorescent staining for ANXA1 (Fig. 5A), which corresponds to the constitutive expression of this protein. In cells treated with Dex or $E_2\beta$, for 12 h, there was a marked increase of the fluorescent staining for total ANXA1 (Fig. 5B,C). A similar increase of the total ANXA1 immunoreactivity was also observed in cells treated with dBcAMP for the same time period (Fig. 5D). Taken together, these results show that Dex, $E_2\beta$ and dBcAMP increase the total cellular content of ANXA1, indicating that these compounds induce the expression of ANXA1 in CCRF-CEM cells.

To investigate whether cAMP mediates Dex-induced and $E_2\beta$-induced ANXA1 expression, the cells were pre-treated with Rp-8-Br-cAMPS (500 μM), for 1 h, before the addition of those stimuli, for another 12 h. The results presented in Fig. 5E–G show that in cells pre-treated with Rp-8-Br-cAMPS the fluorescent staining for ANXA1 was similar to that of control cells, indicating that this cAMP antagonist is effective in preventing the synthesis of ANXA1 induced by either Dex, $E_2\beta$ or dBcAMP. Rp-8-Br-cAMPS alone had no effect on the basal immunoreactivity for ANXA1 (data not shown).

The p38 MAPK inhibitor SB203580 prevents Dex-induced, $E_2\beta$-induced and dBcAMP-induced CREB activation and ANXA1 expression

To further elucidate the mechanism by which Dex, $E_2\beta$ and dBcAMP induce CREB activation and ANXA1 expression, we studied the involvement of the p38 MAPK on this pathway. For this purpose we used SB203580, which is a pyridinyl imidazole compound shown to specifically inhibit the activity of the p38 MAPK.[41–43] SB203580 (40 μM) did not reduce the
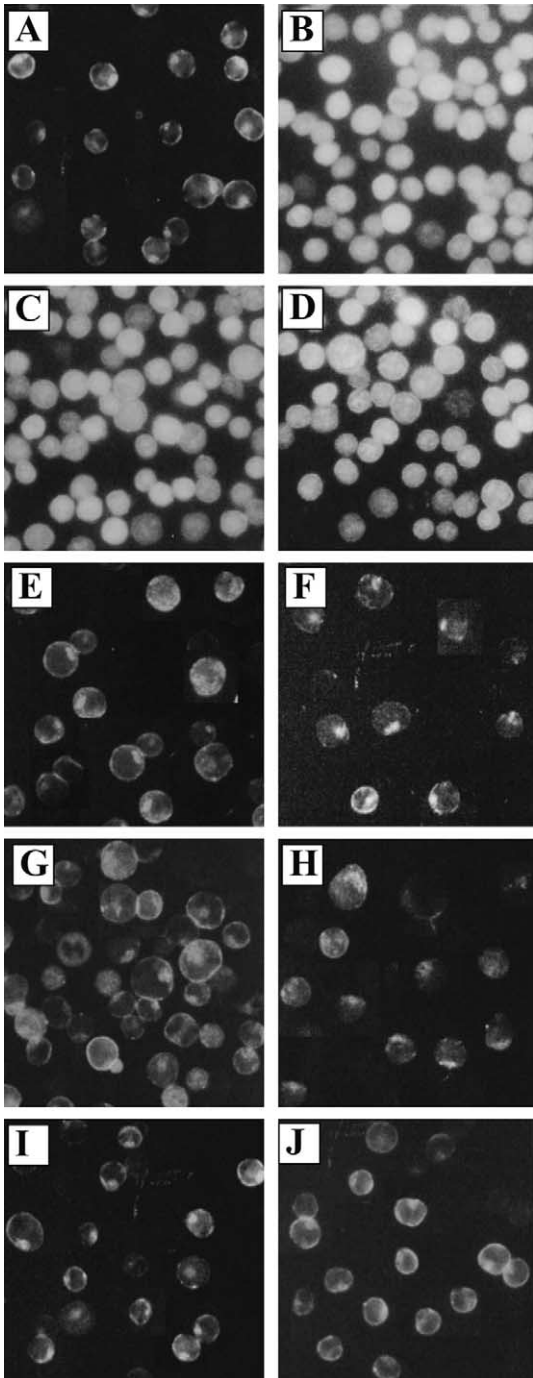
FIG. 5. Rp-8-Br-cAMPS and SB203580 inhibit the expression of ANXA1 induced by Dex, E$_2\beta$ or dBcAMP in CCRF-CEM cells. The cells were pre-treated with Rp-8-Br-cAMPS (500 μM) or with SB203580 (40 μM), for 1 h, before the addition of Dex (1 μM), E$_2\beta$ (1 μM) or dBcAMP (1 mM), for 12 h. The detection of total ANXA1 was performed by immunocytochemistry, as described in Materials and methods. (A) control, untreated cells; (B) cells treated with Dex; (C) cells treated with E$_2\beta$; (D) cells treated with dBcAMP; (E) cells treated with Rp-8-Br-cAMPS plus Dex; (F) cells treated with Rp-8-Br-cAMPS plus E$_2\beta$; (G) cells treated with Rp-8-Br-cAMPS plus dBcAMP; (H) cells treated with SB203580 plus Dex; (I) cells treated with SB203580 plus E$_2\beta$; (J) cells treated with SB203580 plus dBcAMP. The results shown are representative of three independent experiments.

viability of the CCRF-CEM cells, as assessed by the MTT reduction assay (data not shown), indicating the

absence of toxic effects in the experimental conditions used.

To study the involvement of the p38 MAPK on CREB activation, CCRF-CEM cells were pre-treated with SB203580 (40 μM), for 1 h, before the addition of Dex (1 μM), E$_2\beta$ (1 μM) or dBcAMP (1 mM), and then further incubated for the optimal time periods previously identified for each stimulus (Figs. 1–3). The corresponding nuclear extracts were then subjected to EMSA analysis. The results presented in Fig. 6 show that pre-treatment of the cells with SB203580 was effective in preventing CREB activation induced by either Dex, E$_2\beta$ or dBcAMP, as indicated by the reduced intensity of the bands corresponding to the CREB specific complexes (lanes 3, 5 and 7). The involvement of the p38 MAPK on ANXA1 expression, induced by Dex, E$_2\beta$ or dBcAMP, in CCRF-CEM cells was also evaluated by immunocytochernistry. The results showed that pre-treatment of the cells with SB203580 (40 μM), for 1 h, prior to the addition of Dex (1 μM), E$_2\beta$ (1 μM) or dBcAMP (1 mM), for another 12 h, effectively inhibited the increase in the fluorescent staining for total ANXA1 (Fig. 5H–J), as
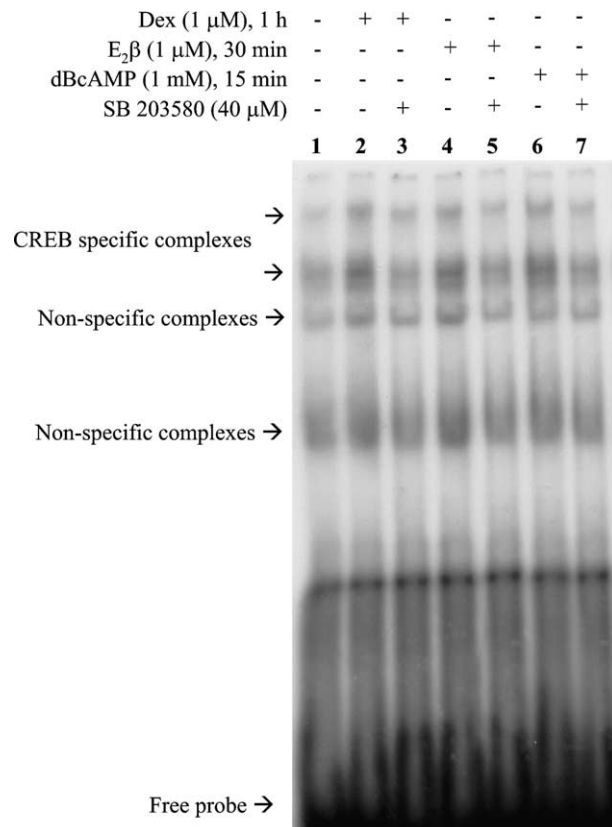
FIG. 6. SB203580 inhibits Dex-induced, E$_2\beta$-induced and dBcAMP-induced CREB activation in CCRF-CEM cells. The cells were incubated in the presence or in the absence (control, lane 1) of the p38 inhibitor, SB203580 (40 μA), for 1 h. Then, the cells were further incubated with Dex (1 μM), E$_2\beta$ (1 μM) or dBcAMP (1 mM), for 1 h, 30 min or 15 min, respectively. Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. The results shown are representative of three independent experiments.

compared with cells treated with Dex, $E_2\beta$ or dBcAMP in the absence of the inhibitor (Fig. 5 B−D).

SB203580 alone had no effect on either the basal activation of CREB, or the basal expression of ANXA1 (data not shown).

Discussion

In this work, we found that both Dex and $E_2\beta$ induce CREB activation, and that the cAMP analogue dBcAMP is as effective as Dex and $E_2\beta$ in activating this transcription factor and in inducing ANXA1 synthesis, in CCRF-CEM cells. We also found that both the cAMP antagonist Rp-8-Br-cAMPS, and the p38 MAPK inhibitor SB 203580 effectively prevent CREB activation and ANXA1 synthesis induced by either Dex, $E_2\beta$ or the cAMP analogue dBcAMP. These results indicate that cAMP and the p38 MAPK are both required for Dex-induced and $E_2\beta$-induced CREB activation and ANXA1 synthesis, and are also strong evidence that CREB activation is required for ANXA1 synthesis in these cells.

The intracellular signalling pathways involved in the expression of ANXA1 in response to steroid hormones are still incompletely understood. Although some reports show that GC hormones up-regulate ANXA1 synthesis by genomic mechanisms,[2,19] studies in different cells indicate that this effect is independent of the activation of nuclear GC receptors.[20,21,23] In agreement with these latter results, our previous studies also indicate that, in CCRF-CEM cells, ANXA1 synthesis is independent of either the nuclear GC receptors or the oestrogen receptors.[4,16] Recently, it was shown that the gene coding for ANXA1, besides a putative GRE, also possesses a specific consensus binding sequence for CREB that was found to be essential for ANXA1 expression in a human choriocarcinoma-derived cell line.[22]

Many stimuli, acting in a variety of cells, increase the intracellular cAMP concentration, which leads to the activation of CREB by phosphorylation of the serine-133 residue.[26,27] The results presented here show that Dex and $E_2\beta$ were as effective as the cAMP analogue dBcAMP in activating CREB (Figs. 1−3). Although Dex and $E_2\beta$ mimicked the effects of dBcAMP, both hormones required longer incubation periods for maximal response, suggesting that cAMP has to be produced, in response to both steroids, before CREB activation can occur. In fact, these findings are in agreement with studies in other cell types showing that Dex and $E_2\beta$ induce the production of cAMP, with variable kinetics, depending on the cell type.[30−33] To further elucidate the role of cAMP on Dex-induced and $E_2\beta$-induced CREB activation, we used the metabolically stable cell-permeant cAMP antagonist Rp-8-Br-cAMPS.[40] Our results show

that when CCRF-CEM cells were pre-treated with the cAMP antagonist, the activation of CREB, in response to either Dex, $E_2\beta$ or dBcAMP, was efficiently prevented (Fig. 4). Taken together, these results indicate that, in CCRF-CEM cells, Dex-induced and $E_2\beta$-induced CREB activation is mediated by cAMP. Effects of Dex and $E_2\beta$ mediated via cAMP, and involving probably non-genomic mechanisms, have also been reported in other cell types.[25,31,44,45]

The results presented in this work (Fig. 5A−C) and in our latest works[4,16] show that, in CCRF-CEM cells, Dex and $E_2\beta$ induce the synthesis of ANXA1. Additionally, we found that treatment of the cells with dBcAMP was as effective as Dex and $E_2\beta$ in inducing ANXA1 synthesis (Fig. 5A−D). Furthermore, the cAMP antagonist Rp-8-Br-cAMPS effectively prevented ANXA1 synthesis in response to either one of those stimuli (Fig. 5E−G). Hence, these results indicate that cAMP is also part of the signaling pathway that leads to ANXA1 synthesis in CCRF-CEM cells stimulated with either Dex or $E_2\beta$. Moreover, the ability of Rp-8-Br-cAMPS to inhibit both CREB activation and ANXA1 expression, induced by either Dex, $E_2\beta$ or dBcAMP, strongly suggests that CREB is required for and mediates ANXA1 transcription in response to those stimuli. The ability of dBcAMP to activate both CREB and ANXA1 synthesis further supports this conclusion.

Members of the MAPK family, namely the p38 MAPK, have been implicated in the phosphorylation and subsequent activation of CREB in response to several stimuli.[28,29,46] On the contrary, some reports indicate that Dex and $E_2\beta$ can up-regulate p38 MAPK activity.[36−38] To further elucidate the signaling mechanisms involved in Dex-induced and $E_2\beta$-induced CREB activation and ANXA1 synthesis, we evaluated the role of the p38 MAPK in those processes by using the pyrydinyl imidazole compound SB203580, which is a specific inhibitor of this kinase.[41−43] The results obtained show that SB203580 effectively prevented the activation of CREB (Fig. 6) and the synthesis of ANXA1 (Fig. 5H−J) induced by Dex, $E_2\beta$ and dBcAMP. Therefore, the activity of the p38 MAPK seems to be indispensable for CREB activation and ANXA1 synthesis to occur in response to Dex, $E_2\beta$ and dBcAMP. These results also indicate that CREB mediates ANXA1 expression induced by either one of these stimuli, in CCRF-CEM cells.

The involvement of CREB on ANXA1 expression induced by Dex, $E_2\beta$ or dBcAMP is further supported by the observation that two different agents, SB203580 and Rp-8-Br-cAMPS, acting by distinct mechanisms, were equally effective in preventing these processes. Furthermore, since SB203580 was also effective in preventing dBcAMP-induced CREB activation and ANXA1 synthesis, these results also indicate that, in CCRF-CEM cells, the p38 MAPK mediates those cAMP-induced responses. These re-

sults are in agreement with several studies that indicate the p38 MAPK can be a downstream target of cAMP, thus participating in cAMP-dependent gene transcription, although the nature of the kinases that link cAMP to the p38 MAPK are still unknown.[47,48]

The ability of Dex and E$_2$β to activate CREB through a signalling pathway involving cAMP and the p38 MAPK, as we observed in the present study, may also be implicated in other effects of GC hormones, besides ANXA1 synthesis. For instance, the p38 MAPK and the cAMP-dependent PKA are required for COX-2 expression in various cell types.[49–51] Therefore, it would be possible that these hormones could also induce COX-2 expression in CCRF-CEM cells. Although we did not address this question directly, various studies have shown that GC hormones, namely Dex, reduce COX-2 expression, which, at least in some of those cells, is mediated by ANXA1.[6–8] The ability of Dex to inhibit COX-2 expression can be an important mechanism underlying the anti-inflammatory actions of GC and their inhibitory effect on cell proliferation. In fact, it was shown that the ability of Dex to inhibit the synthesis of prostaglandins by COX-2 is mediated by ANXA1, leading to the inhibition of cell proliferation.[8] On the contrary, E$_2$β seems to have a dual role on COX-2 expression. In high concentrations, as those used in the present work, this hormone negatively modulates COX-2 expression, whereas in lower concentrations it can up-regulate COX-2 synthesis.[52,53] Regardless of these discrepancies, several studies indicate that other transcription factors besides CREB, namely nuclear factor-κB, are required for COX-2 synthesis in different cells and in response to a variety of stimuli.[50,55] Interestingly, we have recently observed that Dex (1 μM) inhibits nuclear factor-κB activity in CCRF-CEM cells,[55] which suggests that, although activating CREB, Dex and E$_2$β cannot induce COX-2 expression in CCRF-CEM cells.

We have previously observed that neither the GC antagonist RU486 nor the oestrogen antagonist ICI 182,780 can prevent the synthesis of ANXA1, in response to Dex and E$_2$β, respectively, in CCRF-CEM cells.[4,16] Thus, the ability of Dex and E$_2$β to induce ANXA1 synthesis seems to be independent of genomic mechanisms, mediated by the classical GC and oestrogen receptors, respectively. Instead, this action of both steroids may involve their binding to membrane GC or oestrogen receptors[12,56–58] or alternatively, it may be mediated by non-specific interactions with the plasma membrane of CCRF-CEM cells.[59,60]

In summary, the results presented here suggest that, in CCRF-CEM cells, Dex-induced and E$_2$β-induced ANXA1 expression requires the activation of the transcription factor CREB, which in turn seems to be mediated by cAMP and the p38 MAPK. These findings also suggest that, besides the nuclear steroid hormone receptors, other transcription factors, namely CREB, may play important roles in mediating the anti-inflammatory actions of GC and oestrogen hormones.

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