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Expression of Annexin I, II, V, and VI by Rat Osteoblasts in Primary Culture: Stimulation of Annexin I Expression by Dexamethasone

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ABSTRACT

To determine whether rat osteoblasts synthesize proteins of the annexin family and to evaluate the extent to which glucocorticoids modulate the expression of annexins by these cells, osteoblasts were grown in primary cultures in the absence or presence of dexamethasone, and the expression of annexins was evaluated by immunoblotting using polyclonal antibodies against human annexins. Four different annexins (I, II, V, and VI) were found to be expressed by rat osteoblasts. The expression of annexin I, but not the other annexins studied, was increased in osteoblasts cultured in the presence of dexamethasone (173 ± 33% increase comparing untreated cells and cells treated for 10 days with 5×10^{-7} M dexamethasone). Increased expression of annexin I was observed after the third day of exposure to dexamethasone and rose thereafter until day 10; annexin I expression increased with dexamethasone concentrations above 10^{-10} M throughout the range of concentrations studied. The increase in annexin I protein was associated with an increase in annexin I mRNA and was completely blocked by the concomitant addition of the glucocorticoid receptor antagonist RU 38486. The increase in annexin I content following dexamethasone treatment was associated with an increase in alkaline phosphatase activity and PTH-induced cAMP stimulation, whereas phospholipase A_2 activity in the culture medium was reduced to undetectable levels. The finding that four annexins are expressed in rat osteoblasts in primary culture raises the possibility that these proteins could play an important role in bone formation by virtue of their ability to bind calcium and phospholipids, serve as Ca^{2+} channels, interact with cytoskeletal elements, and/or regulate phospholipase A_2 activity. In addition, the dexamethasone-induced increase in annexin I may represent a mechanism by which glucocorticoids modify osteoblast function.

INTRODUCTION

THE PRODUCTION OF A MINERALIZED ORGANIC MATRIX by the osteoblasts depends on a series of complex events, including the synthesis of matrix proteins, the specific organization of this matrix necessary to create a milieu propitious for the deposition of mineral, and subsequent mineral deposition.[1,2] A number of osteoblast products and bone matrix components necessary for bone formation have been characterized, including bone sialoproteins I and II (cell attachment),[3,4] bone morphogenic proteins (promotion of bone formation),[5] alkaline phosphatase and osteonectin (bone mineralization),[6,7] and osteocalcin (bone remodeling).[8] Although studies evaluating the specific function of these components in the production of the mineralized organic matrix have improved our understanding of this process, important gaps remain in our knowledge of bone formation. In particular the definition of osteoblast products necessary for the initiation of bone mineralization is far from complete.

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In recent years, independent approaches have led to the identification of a family of proteins, "annexins," which are characterized by their ability to bind calcium and interact with phospholipids in a calcium-dependent fashion.[9-11] The annexins (originally designated lipocortin, calpactin, endonexin, and chromobindin)[12] are structurally related and appear to be derived from a common ancestral gene.[9] Annexins are present in a number of different tissues, including cartilage and matrix vesicles isolated from chondrocytes.[13-15] Although the presence of annexins in bone or bone cells in culture has not been reported, these proteins could play a role in mineralization by virtue of their ability to bind to calcium and phospholipids. For example, calcium acid phospholipid complexes have been found to be concentrated in mineralizing connective tissue and have been found to promote hydroxyapatite deposition.[16-18] Other roles for annexins in the homeostasis of bone are also possible. The phenotype of osteoblasts is subject to regulation by glucocorticosteroids, which are potent modulators of bone cell metabolism and function in vivo[19] and in vitro.[20-22] One member of the annexin family (lipocortin I) was defined as a glucocorticoid-inducible phospholipase A_2 inhibitor.[23-25] In this context, some of the effects of glucocorticoids on bone, including their effects on arachidonic acid metabolism by osteoblasts, could be mediated through the regulation of such annexins.

The purpose of this study was threefold. First, we determined whether osteoblasts in primary culture synthesize members of the annexin family. Second, we evaluated the extent to which glucocorticosteroids modulate the expression of annexins by these cells, and compared these changes to those observed in two other characteristics of osteoblast activities, parathyroid hormone (PTH)-induced cAMP production and alkaline phosphatase activity. Finally, in an attempt to identify the mechanism by which glucocorticosteroids modulate arachidonic acid metabolism in osteoblasts, we sought evidence that annexins modify osteoblast phospholipase A_2 activity.

MATERIALS AND METHODS

Primary rat osteoblast cultures

Osteoblasts were isolated from the calvariae of newborn rats (Charles River, Saint Aubin les Elbeuf, France) by sequential enzymatic digestion, as previously described for mice,[26] with slight modifications. The central parts of parietal bones were removed aseptically and the periosteal layers carefully stripped off. Parietal bones were extracted by stirring for 15 minutes at 37°C in Ca-free phosphate-buffered saline containing 5 mM EDTA and 0.05% trypsin, followed by three incubations of 20 minutes in phosphate-buffered saline containing 0.1% collagenase (Sigma, St. Louis, MO). The cells released during the last two incubations were combined and cultured. Cells were plated at 2×10^4 cells/cm^2 in 0.25 ml/cm^2 of Dulbecco's modified Eagle's medium (Eurobio, les Ullis, France) supplemented with 100 U/ml of penicillin G, 50 μg/of streptomycin, 1 mM glutamine (complete medium), and 20% fetal calf serum (FCS) and cultured at 37°C in 95% air and 5% CO_2. The medium was replaced 48 h later and every third day thereafter with complete medium containing 10% FCS. Cells reached confluence on day 4-5 of culture.

Pretreatment of the cells

Dexamethasone (Sigma; 5 μl/ml culture medium, 5×10^{-12} to 5×10^{-6} M final concentration) was added to osteoblast cultures for various times. Fresh dexamethasone was added at each medium change. In some experiments, 50 μl/ml culture medium of 1×10^{-4} M RU 38486 (kindly provided by Roussel Uclaf Laboratories, Romainville, France, 5×10^{-6} M final concentration) was added to the cultures in addition to dexamethasone. Stock solutions of dexamethasone and RU 38486 (10^{-2} M in ethanol) were stored at −20°C, and intermediate dilutions were performed in medium without serum. Control cultures, maintained in parallel, received 5 or 55 μl solvent alone (ethanol diluted 1:100 in medium without serum).

Sodium dodecyl:sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting

Cells were grown in 28 cm^2 culture dishes and treated with dexamethasone as described. To collect the cells, culture dishes were placed on ice, washed twice with 3 ml ice-cold Dulbecco's phosphate-buffered saline, scraped in 200 μl Laemmli SDS-sample buffer,[27] and immediately boiled for 10 minutes. Samples were stored at −20°C. The protein content was determined on cells grown in dishes of the same size and cultured in parallel. Protein (100 μg) was loaded on SDS-polyacrylamide gels.

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli[27] on 12.5% acrylamide gels (1.5 mm thick). The molecular weight of proteins was determined by comparison to migration of standard proteins (Pharmacia-LKB), which were electrophoresed in parallel. The separated proteins were then transferred from the gel to nitrocellulose membranes (HAHY 304 FO, Millipore) according to Burnette[28] using an electroblotting apparatus (Pharmacia-LKB 2117-250 Novablot) operated at constant current (250 mA) for 2.5 h in 20 mM Tris-base, 150 mM glycine and 20% methanol. At the end of the transfer, nitrocellulose membranes were stained with india ink (1 μl india ink per ml of 50 mM Tris and 150 mM NaCl) or with 0.2% Ponceau red (Serva, 33429) in 3% trichloroacetic acid for 10 minutes and incubated in 50 mM Tris, 150 mM NaCl, and 5% skimmed powdered milk (TNM) for 1 h at 37°C with constant shaking. Antisera against annexins (see later) were diluted (1:500) in 10 ml TNM and incubated with membranes for 15 h at 4°C with constant shaking. Following five washes in TNM, the blot was incubated for 30 minutes at room temperature with ^{125}I-labeled protein A diluted in 15 ml TNM (10^5 cpm/ml, specific activity ≅24 mCi/mg), washed four more times with TNM, and dried between sheets of filter paper; autoradiography of the dried blot was performed for 4-7 days at −80°C. Strips of the blots that displayed bands on the autoradiograms were excised and the amount of radioactivity was quantified

using a gamma counter. Radioactivity measured in strips of the blots not displaying bands was subtracted as background.

Polyclonal antibodies were raised in rabbits by popliteal lymph node injection[29] of annexins I, V, and VI purified from human placenta.[30,31] Cross-reactivity between the different antisera was less than 5%. Antibody against annexin II was a gift from J.C. Cavadore (INSERM U 249, CNRS LP 8402, Montpellier, France).

Northern blot analysis

Cells were grown in 69 cm^2 culture dishes and treated with dexamethasone as described. Total cellular RNA was isolated with guanidinium thiocyanate followed by centrifugation in cesium chloride gradients.[32] RNA (10 μg) was electrophoresed in a 1% agarose gel containing 0.45 M formaldehyde[32] in 20 mM MOPS (3-(N-morpholino) propane sulfonic acid) buffer at 160 V for 1 h, transferred to Nytran nylon membranes (Schleicher and Schuell, NY), and dried for 2 h at 80°C. Blots were hybridized with cDNA of human annexins I and VI (kindly provided by Barbara Wallner, Biogen Research Corporation, Cambridge, MA), which were labeled by random priming (1.5×10^7 dpm/25 ng cDNA, 10^6 cpm/ml) and denatured by heating to 100°C for 10 minutes before use. Prehybridization (30 minutes at 65°C) and hybridization (overnight, 65°C) were done in 50 mM PIPES, pH 6.5, 50 mM sodium phosphate, 1 mM EDTA, 100 mM NaCl and 5% SDS.[33] Blots were then washed in standard saline citrate (SSC) and 5% SDS, first in 20 ml for 20 minutes at room temperature and again in 400 ml for 30 minutes at 65°C. To ensure that the total amount of RNA transferred was similar for each sample, the blots were hybridized with γ^{32} P-labeled 28*S* RNA probe as described by Barbu and Dautry.[34] Blots were then dried and autoradiographed for 24 h.

Measurement of phospholipase A_2 activity

Cells were grown in 69 cm^2 culture dishes and treated with dexamethasone as indicated earlier. After 10 days in culture, medium was collected, cells were scraped in 2 ml culture medium without serum, and both medium and cells were stored at −20°C. On the day of the assay, cells were lysed at 4°C by ultrasonication (30 s, 150 W) using an MSE sonifier. Phospholipase A_2 activity was measured in culture medium and cell homogenates using a fluorometric assay as described by Radvanyi et al.[35] The fluorescent substrate [1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-monomethyl phosphatidic acid; Interchim, Montluçon, France] was dried under nitrogen and suspended in ethanol at 0.2 mM. Vesicles were prepared by mixing the ethanol solution of fluorescent phospholipid with an aqueous medium for 2 minutes. The reaction solution was prepared by introducing sequentially into a 4 × 10 mm disposable plastic cuvette 980 μl buffer (100 mM Tris-HCl, 1 M NaCl, and 1 mM EGTA, pH 7.5), 10 μl substrate (2 μM final concentration), and 10 μl of a 10% fatty acid-free bovine serum albumin solution (0.1% final concentration); aliquots of cellular homogenates or medium (10–50 μl) were introduced into the cuvettes and allowed to equilibrate at 37°C for 1 minute. The reactions were then initiated by the addition of $CaCl_2$ at 10 mM (final concentration). The fluorescence measurements were performed with a Jobin et Yvon JY3D spectrofluorometer equipped with a Xenon lamp. The fluorescence intensity was monitored using excitation and emission wavelengths of 345 and 398 nm, respectively, and a slit width of 4 nm. The final ethanol concentration was less than 0.1% and had no effect on the assay. Phospholipase A_2 activity measured in medium without serum and in medium containing 10% FCS was used as a blank for phospholipase A_2 activity in cells and medium, respectively. Results are expressed as pmol hydrolyzed substrate/minute/mg protein.

cAMP cellular content

Cells were grown in 24-well culture plates and treated for 10 days with dexamethasone as before. Stimulation of cAMP production was performed for 10 minutes at room temperature in the presence of 1 mM isobutyl-1-methylxanthine (Sigma) and extracted with ethanol as previously described.[36] cAMP production was determined as previously described[36] using a modification of the Gilman pro-

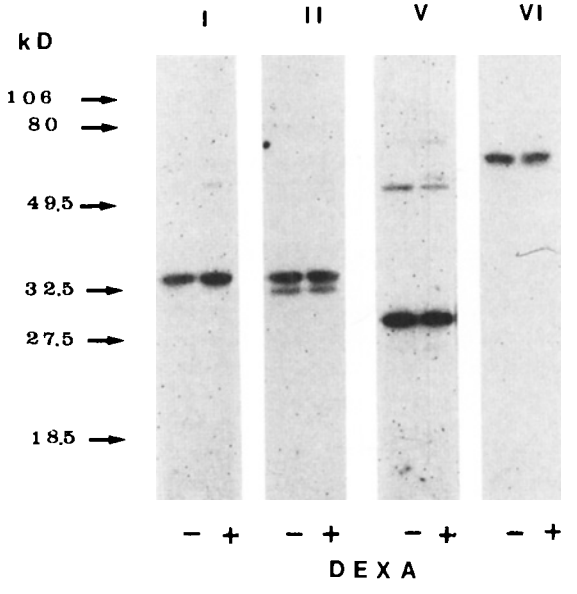

FIG. 1. Expression of annexin I, II, V, and VI in osteoblasts. Effect of dexamethasone. Western blots of annexin I, II, V, and VI were detected by Western blotting in protein extracts from osteoblasts cultured in the absence (−) or in the presence (+) of 5×10^{-7} M dexamethasone for 10 days. Osteoblast cultures, dexamethasone treatment, SDS-polyacrylamide gel electrophoresis, and Western blot analysis of annexins were performed as described in Materials and Methods. Protein (100 μg) was electrophoresed in each lane. In addition to annexin V, material with a higher molecular weight (MW) was recognized by the annexin V antibodies. The MW of this cross-reactive material does not correspond to that of any annexin described, and we have no indication of its nature. The intensity of this band was very low compared to that given by annexin V. The data reported are representative of two independent experiments.

tein binding assay[37] in which human erythrocytes are used as the source of the cAMP binding protein.[38] Results are expressed as picomoles cAMP/100 μg protein.

Cellular alkaline phosphatase activity

Cells were grown in 12-well culture plates and treated with dexamethasone as described for 2-10 days. At the end of the culture period, medium was aspirated and the plates were stored at −20°C. On the day of the assay, cells from each dish were scraped into 1 ml freshly prepared solution containing 8.5 g saccharose and 100 μl Triton X-100/100 ml water. Cells were sonicated for 20 s on ice and centrifuged for 10 minutes at 2500 rpm at 4°C, and alkaline phosphatase activity was measured in the supernatant using *p*-nitrophenyl phosphate as a substrate (Sigma). Aliquots (100 μl) of supernatant were incubated for 1 h at 37°C in the presence of 1 μmol substrate, 0.625 μmol $MgCl_2$, and 25 μmol glycine in a total volume of 500 μl at pH 10.5. The reaction was stopped by adding 2.5 ml 1 N NaOH and 10 mM EDTA. The amount of *p*-nitrophenol liberated was measured by absorbance at 405 nm. Results are expressed as nmol *p*-nitrophenol released/minute/100 μg protein. Preliminary experiments demonstrated that alkaline phosphatase activity could not be detected in the culture medium.

Protein content

Protein content was estimated by the method of Lowry et al.[39] using bovine serum albumin as standard.

Presentation of data

Results are expressed as mean ± standard deviation unless otherwise stated. All immunoblots and Northern blots were performed with equivalent amounts of cellular proteins and total RNA, 100 and 10 μg, respectively.

RESULTS

Expression of annexins I, II, V, and VI in osteoblasts: Effect of dexamethasone

Cellular proteins were extracted from rat osteoblasts and separated by electrophoresis and expression of annexins evaluated by immunoblotting. Proteins reactive with antisera specifically recognizing annexins I, II, V, and VI were expressed by these cells (Fig. 1). In each case, the molecular weights of these proteins corresponded closely to those previously reported for these four different annexins. In some cases, fainter bands of lower molecular weight were also reactive with these antibodies and presumably represented annexins that were partially degraded during the extraction procedures.

The expression of annexin I was significantly increased in osteoblasts cultured for 10 days in the presence of 5×10^{-7} M dexamethasone (Fig. 1). Quantitation of annexin I expression by determining ^{125}I-labeled protein A bound to Western blots demonstrated a 173 ± 33% increase comparing dexamethasone-treated and untreated cells ($n = 7$). In contrast, dexamethasone treatment did not significantly change expression of annexin II, V, or VI (Fig. 1).

Time course and dose-response curve of the induction of annexin I by dexamethasone

The characteristics of the dexamethasone-induced stimulation of annexin I were further investigated by determining the kinetics and the dose-response curve of the dexamethasone effect. An increase in annexin I expression was not observed during the first 3 days of exposure of osteoblasts to 5×10^{-7} M dexamethasone (Fig. 2). Increased expression of annexin I was observed after the third day of exposure and thereafter rose progressively until day 10, the latest time point evaluated.

When osteoblasts were cultured in the presence of dexamethasone at concentrations $< 10^{-10}$ M, no increase in annexin I expression was observed after 10 days (Fig. 3). In

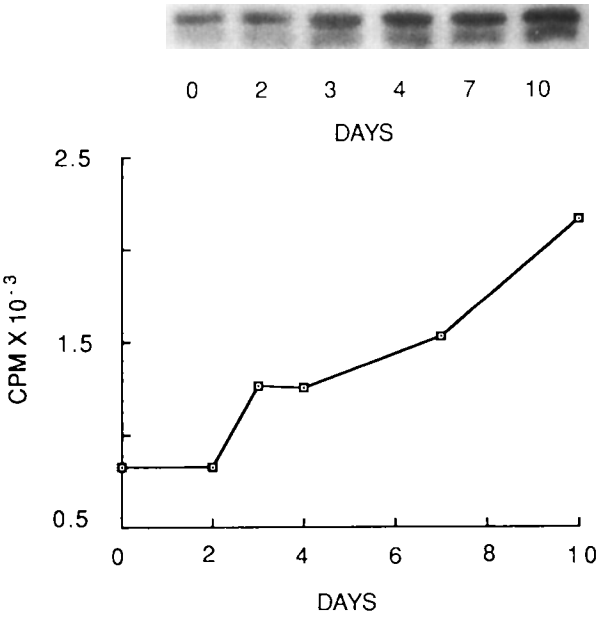

FIG. 2. Time course of the stimulation by dexamethasone of annexin I content in osteoblasts. Western blot analysis of annexin I (top) was performed in protein extracts from osteoblasts cultured in the presence of 5×10^{-7} M dexamethasone for the indicated times. The bands of lower molecular weight are likely to be proteolytic fragments of annexin I generated during the cell preparation for the protein blotting. Regions of the blots corresponding to annexin I, including the lower molecular weight bands, were excised, and the amount of radioactivity in each band was determined in a gamma counter (bottom). Osteoblast cultures, dexamethasone treatment, SDS-polyacrylamide gel electrophoresis, and Western blot analysis of annexin I were performed as described in Materials and Methods. Protein (100 μg) was electrophoresed in each lane. Similar results were obtained in two independent experiments.

general, annexin I expression increased with dexamethasone concentrations above 10^{-10} M throughout the range of concentrations studied (Fig. 3).

In some experiments, the glucocorticoid receptor antagonist RU 38486 was added to the cultures in the presence or in the absence of dexamethasone to determine if the effect of dexamethasone was mediated through the activation of glucocorticoid receptors. The induction of annexin I by dexamethasone (5×10^{-7} M, 10 days) was totally blocked by the concomitant addition of 5×10^{-6} M RU 38486 (Fig. 4). RU 38486 alone had no effect on annexin I content (Fig. 4).

Effect of dexamethasone on annexin mRNA

To determine if the increase in annexin I protein induced by dexamethasone was associated with a similar increase in annexin I mRNA, cells were maintained for 7 and 10 days in the presence or absence of 5×10^{-7} M dexamethasone and the level of annexin I mRNA was examined by Northern blot analysis. Results demonstrated that the increase in annexin I protein was associated with an increase in annexin I mRNA, comparing dexamethasone-treated and untreated osteoblasts (Fig. 5). In agreement with the absence of effect of dexamethasone on the content of annexin VI in osteoblasts, no effect of dexamethasone on annexin VI mRNA was observed (Fig. 5).

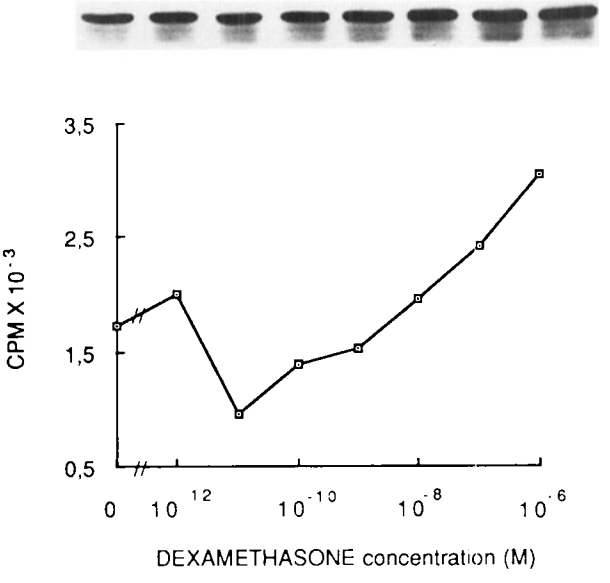

FIG. 3. Annexin I content in osteoblasts as a function of increasing concentrations of dexamethasone. Western blot analysis of annexin I (top) was performed in protein extracts from osteoblasts cultured in the presence of the indicated dexamethasone concentrations for 10 days. Regions of the blots corresponding to annexin I were excised, and the amount of radioactivity in each band was determined in a gamma counter (bottom). Osteoblast cultures, dexamethasone treatment, SDS-polyacrylamide gel electrophoresis, and Western blot analysis of annexin I were performed as described in Materials and Methods. Protein (100 μg) was electrophoresed in each lane. The data reported are representative of two independent experiments.

Effect of dexamethasone on other osteoblast phenotypic characteristics

Alkaline Phosphatase Activity: Treatment of the cells with 5×10^{-7} M dexamethasone for 10 days resulted in an increase in alkaline phosphatase activity from 0.94 ● 0.23 to 2.21 ± 0.10 nmol/minute/100 μg protein (mean ● SD, $n = 6$; Fig. 6). Like the increase in annexin I, maximal increase in alkaline phosphatase was observed at 10 days, the latest time studied, whereas shorter exposures had little or no effect on alkaline phosphatase activity.

PTH-Induced cAMP Production: Treatment of the cells with 5×10^{-7} M dexamethasone for 10 days also greatly enhanced the stimulation of cAMP production by PTH (14- and 106-fold stimulation in response to 240 nM PTH, respectively, without and with dexamethasone treatment; Fig. 7).

Phospholipase A_2 Activity in Osteoblast Cultures: Because annexin I was first identified by its ability to inhibit phospholipase A_2, it was of interest to measure phospholipase A_2 in osteoblasts cultured in the absence or presence of dexamethasone. Phospholipase A_2 activity was easily detectable in both culture supernatants and cell sonicates of osteoblasts maintained in the absence of dexamethasone (Table 1). Interestingly, treatment of cells with 5×10^{-7} M

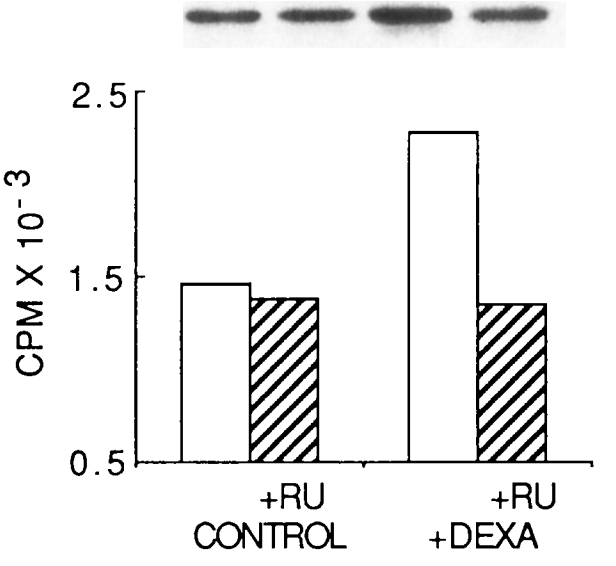

FIG. 4. Effect of RU 38486 on the stimulation by dexamethasone of annexin I expression in osteoblasts. Western blot analysis of annexin I (top) was performed in protein extracts from osteoblasts cultured in the absence (control) or in the presence of 5×10^{-7} M dexamethasone (+Dexa) and 5×10^{-6} M RU 38486 (+RU) for 10 days. Regions of the blots corresponding to annexin I were excised, and the amount of radioactivity in each band was determined in a gamma counter (bottom). Osteoblast cultures, dexamethasone, and RU 38486 treatment, SDS-polyacrylamide gel electrophoresis, and Western blot analysis of annexin I were performed as described in Materials and Methods. Protein (100 μg) was electrophoresed in each lane. The data reported are representative of two independent experiments.

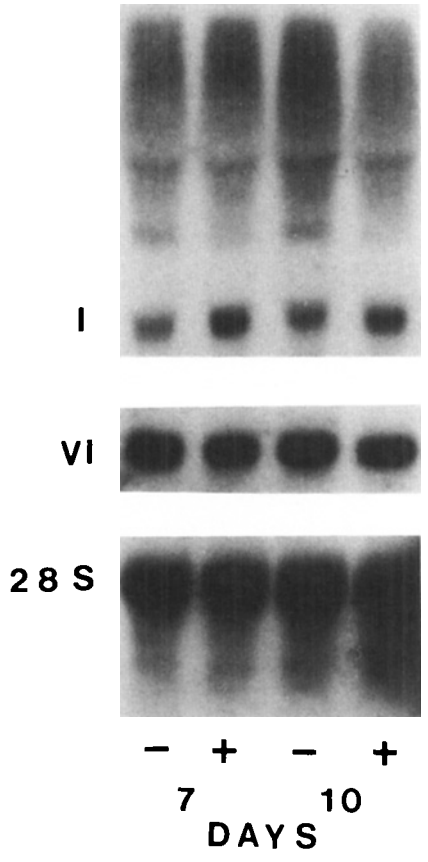

FIG. 5. Northern blot analysis of RNA from osteoblasts probed with cDNA for human annexins I and VI and a 28S ribosomal RNA probe. Osteoblasts were cultured in the presence of 5×10^{-7} M dexamethasone for 7 and 10 days. Total cellular RNA was prepared using a cesium gradient, electrophoresed in a 1% agarose gel and transferred to Nytran nylon membranes, and blots were hybridized with radiolabeled probes as described in Materials and Methods. RNA (10 μg) was electrophoresed in each lane.

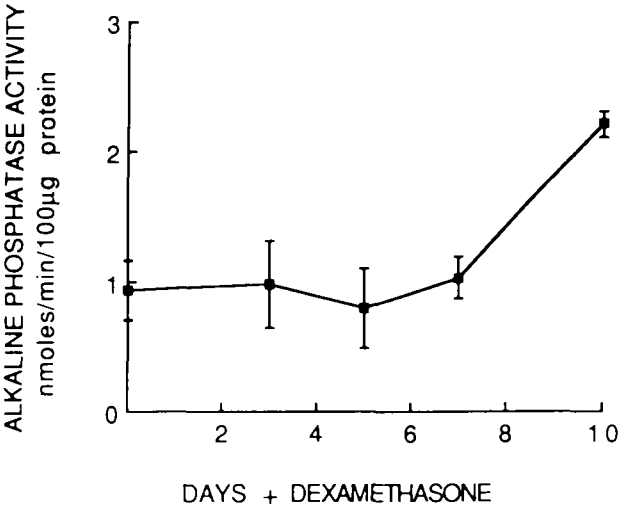

FIG. 6. Effect of dexamethasone on cellular alkaline phosphatase activity in osteoblasts. Osteoblasts were cultured for 10 days without dexamethasone or in the presence of 5×10^{-7} M dexamethasone for the indicated times, and cellular alkaline phosphatase activity was measured as described in Materials and Methods. Alkaline phosphatase activity measured in osteoblasts cultured for 10 days without dexamethasone is the value given at time 0. Results are the mean ± SD, $n = 6$.

dexamethasone for 10 days suppressed phospholipase A_2 activity in the culture medium to undetectable levels, whereas no consistent effect on cellular phospholipase A_2 activity was observed (Table 1).

Total Cellular Protein: Dexamethasone treatment decreased the amount of total cellular protein in the cultures (35 ± 6 and 24 ± 5 μg/cm², respectively, without and after dexamethasone treatment; $n = 5$, $p < 0.05$). The electrophoretic pattern of proteins obtained from osteoblasts cultured in the absence of dexamethasone and after treatment with 5×10^{-7} M dexamethasone for 10 days appeared similar both before and after transfer to nitrocellulose membranes, as demonstrated by the Coomassie blue staining of the SDS-polyacrylamide gels and the india ink staining of nitrocellulose membrane after transfer (Fig. 8).

DISCUSSION

This study demonstrates that four members of the annexin family, annexins I, II, V, and VI, are produced by rat osteoblasts maintained in primary cultures, and it is the first description of the expression of annexins by bone cells. Exposure of osteoblasts to 5×10^{-7} M dexamethasone results in an increase in the cellular content of annexin I and mRNA coding for this protein, whereas the cellular content of the other annexins studied was not modified. The increase in annexin I content following dexamethasone treatment was not an early event, however, but was associated with an increase in other activities considered typical of mature osteoblasts (alkaline phosphatase activity and PTH-induced cAMP stimulation).

Annexins and bone formation

Although annexins share structural and functional features,[9-11] the presence of a unique N-terminal sequence for each annexin suggests that each annexin has a specific role. In view of the previously described properties of different members of the annexin family, several possible mechanisms exist by which annexins could contribute to bone formation, none of which is mutually exclusive.

First, annexins share a 17 amino acid residue consensus sequence that is probably involved in Ca^{2+}-dependent binding to anionic phospholipids.[10] The ability of annexins to bind calcium and phospholipids could contribute to miner-

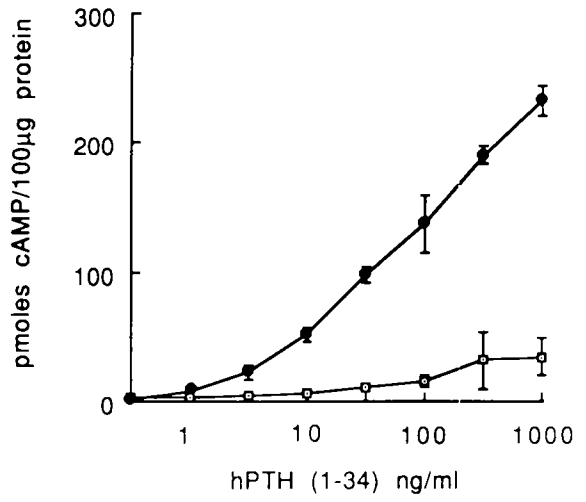

FIG. 7. Stimulation of cAMP production by increasing PTH concentrations in osteoblasts treated or not with dexamethasone. Osteoblasts were cultured in the absence or in the presence of 5×10^{-7} M dexamethasone for 10 days, and stimulation of cAMP production by the indicated concentration of PTH was performed as described in Materials and Methods. Results are the mean ± SD of one representative experiment, performed in triplicate: untreated cells (open squares); dexamethasone-treated cells (closed circles). Similar results were obtained in a second experiment.

TABLE 1. PHOSPHOLIPASE A_2 ACTIVITY IN THE MEDIUM AND CELL PELLET OF OSTEOBLASTS CULTURED IN THE ABSENCE OR PRESENCE OF 5×10^{-7} M DEXAMETHASONE[a]

	Medium		*Cells*	
Experiment	*Control*	*+ Dexa*	*Control*	*+ Dexa*
1	3.9	<0.1	3.3	1.1
2	1.67	<0.1	1.5	1.6

[a]Osteoblasts were cultured in the absence (control) or in the presence of 5×10^{-7} M dexamethasone (+ Dexa) for 10 days, and medium and cellular phospholipase A_2 activity was measured as described in Materials and Methods. Results are the mean of two determinations per experiment and are expressed as pmol hydrolyzed fluorescent substrate [1-palmitoyl-1-(10-pyrenyldecanoyl)*sn*-glycero-3-monomethylphosphatidic acid]/minute/mg protein.

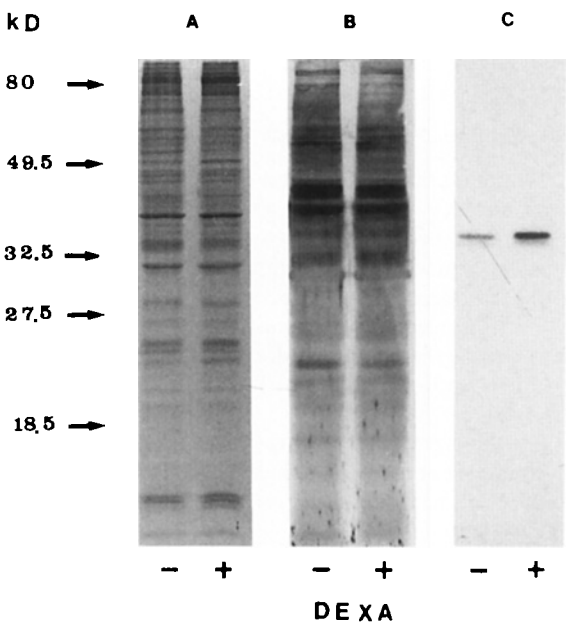

FIG. 8. Distribution of cellular proteins obtained from untreated (−) and dexamethasone-treated (+) osteoblast cultures. Proteins were separated by SDS-polyacrylamide gel electrophoresis. (A) Pattern of proteins as revealed by staining proteins in the gel with Coomassie blue R-250 and (B) proteins after transfer to nitrocellulose membranes and stained with india ink. (C) Western blots of annexin I from the corresponding protein extracts. Osteoblast cultures, dexamethasone treatment, SDS-polyacrylamide gel electrophoresis, and Western blot analysis of annexin I were performed as described in Materials and Methods. Protein (100 µg) was electrophoresed on each lane. A similar pattern was obtained in all experiments.

alization of bone matrix, as was previously suggested for the mineralization of cartilage.[13] In this regard, annexins have been described as complexes to lipids in cartilage matrix vesicles,[14] and calcium acid phospholipid complexes have been shown to promote the deposition of hydroxyapatite in vitro and in vivo.[16-18]

Second, different members of the annexin family have been found to bind to intracellular cytoskeletal elements [e.g., binding of annexins I, II, and VI to actin[25]] and tissue connective components [e.g., binding of annexin V (anchorin CII) to type II collagen[15]]. Furthermore, anchorin CII has been suggested as responsible for fibroblast-collagen interactions,[40] implying that it could bind to collagen types other than collagen type II, in particular collagen type I. Through such interactions, annexins could contribute to a variety of processes required for bone formation, such as budding of matrix vesicles from the plasma membranes of osteoblasts and the attachment of osteoblasts to extracellular collagen.

Third, annexin V has been shown to exhibit Ca^{2+} channel activity,[41] consistent with the idea that annexin V could regulate Ca^{2+} transport in osteoblasts.

Finally, annexins could serve as regulatory proteins. For example, annexin I, previously called lipocortin I, was originally defined as a corticosteroid-inducible phospholipase A_2 inhibitor.[23,24] We demonstrated in this study that phospholipase A_2 activity could be detected in both medium and cell layer of rat osteoblasts. It is possible that the striking reduction in phospholipase A_2 in the medium of cells treated with glucocorticoids resulted, at least in part, from the increase in annexin I observed in these cells. It should be stressed, however, that corticosteroid treatment had no consistent effect on intracellular phospholipase A_2 activity. Further studies are required to determine the role, if any, of annexin I in the regulation of phospho-

lipase A_2 activity and to evaluate the consequences of dexamethasone-induced changes in phospholipase A_2 activity on osteoblast function.

Dexamethasone and annexin I expression

We found that the addition of dexamethasone to osteoblast cultures increased the expression of annexin I, but not of the other annexins, in a time- and concentration-dependent manner. The induction of annexin I by dexamethasone was blocked by the concomitant addition of the glucocorticoid receptor antagonist RU 38486, demonstrating that dexamethasone-induced annexin I RNA and protein synthesis occurred via the activation of the glucocorticoid receptor.

Conflicting data exist in the literature regarding the inducibility of annexin I by glucocorticoids.[23,24] Steroid induction of annexin I has been observed consistently in monocytes incubated in vitro or recovered from animals or humans treated with steroids. In contrast, no induction of annexin I mRNA or protein has been demonstrated in most cell lines, including in U937 cells[42] and primary cultures of human endothelial cells,[43] although dexamethasone was found to increase both annexin I mRNA and protein in 3T3 fibroblasts.[44] The discrepancy observed between these studies may be explained in part by the state of differentiation of the cells[45] and/or by the experimental design used to characterize the effect of dexamethasone. For instance, the effect of dexamethasone on annexin I synthesis in human endothelial cells was studied after 3 h of incubation of the cells in presence of the steroid,[43] but the effect of dexamethasone on 3T3 fibroblasts was observed after 4 days of culture in the presence of dexamethasone.[44] Consequently the term "induction" covers two different processes, a rapid induction, as described in monocytes, and a relatively slow induction generally associated with cellular differentiation, as described in 3T3 fibroblasts. The induction of annexin I content by dexamethasone in osteoblasts was never detected before 3 days of exposure to dexamethasone. The increase in annexin I was associated with an increase in cellular alkaline phosphatase activity and with a potentiation of the stimulation of cAMP production by PTH, as described for human bone cells,[46,47] rodent osteoblastic cells,[48] and rat osteosarcoma cells.[49-52] Stimulation of PTH-induced cAMP production and alkaline phosphatase activity are two hallmarks of the osteoblastic phenotype. Such an effect of dexamethasone on osteoblast differentiation has been observed in studies in which dexamethasone has been shown to stimulate osteogenesis[20,22] and the expression of bone matrix proteins associated with mineral tissue formation.[21] Thus, the induction of annexin I expression by dexamethasone may be part of a general effect of dexamethasone on osteoblast differentiation. It will be interesting to compare the regulation of annexin metabolism in cells cultured in the presence of factors thought to promote matrix deposition and mineralization, such as ascorbic acid and β-glycerophosphate.

In conclusion, we report the expression of annexins I, II, V, and VI by rat osteoblasts in primary culture. Because of their biochemical properties and their specific regulation, this unique group of membrane-directed regulatory proteins are likely to play a significant role in the osteoblast function and bone metabolism. The demonstration that glucocorticoids strongly inhibit phospholipase A_2 activity release in the medium and increase the expression of annexin I may provide new insights into the mechanisms by which glucocorticoids modify osteoblast biology.

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