Journal of Cellular Biochemistry 78:34–46 (2000)

Annexin II Is the Membrane Receptor That Mediates the Rapid Actions of 1α,25-Dihydroxyvitamin D_3

Daniel T. Baran,[1,2,3]* John M. Quail,[1] Rahul Ray,[4] John Leszyk,[5] and Thomas Honeyman[6]

[1]Department of Orthopedics, University of Massachusetts Medical School, Worcester, Massachusetts 01655
[2]Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655
[3]Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655
[4]Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118
[5]Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655
[6]Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Abstract 1α,25-Dihydroxyvitamin D_3 has been shown to exert its effects by both genomic (minutes to hours) and rapid (seconds to minutes) mechanisms. The genomic effects are mediated by interaction with the nuclear vitamin D receptor. We show that the vitamin D analog, [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, is specifically bound to a protein (molecular weight 36 kDa) in the plasma membrane of rat osteoblastlike cells (ROS 24/1). The plasma membrane protein labeled with the bromoacetate analog was identified as annexin II by sequence determination and Western blot. Partially purified plasma membrane proteins (PI 6.9–7.4) and purified annexin II exhibited specific and saturable binding for [^{3}H]-1α,25-dihydroxyvitamin D_3. Antibodies to annexin II inhibited [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate binding to ROS 24/1 plasma membranes, immunoprecipitated the ligand–protein complex, and inhibited 1α,25-dihydroxyvitamin D_3–induced increases in intracellular calcium in ROS 24/1 cells. The results indicate that annexin II may serve as a receptor for rapid actions of 1α,25-dihydroxyvitamin D_3. J. Cell. Biochem. 78:34–46, 2000.

Key words: annexin II; membrane receptor; 1,25-dihydroxyvitamin D_3; nongenomic actions, cell calcium

1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) is the most potent vitamin D metabolite. Specific nuclear vitamin D receptors (nVDR) for this secosteroid have been found in numerous organs and cell lines [Minghetti and Norman, 1988]. The binding of 1α,25-$(OH)_2D_3$ to the nVDR and subsequent binding of the hormone–receptor complex to specific DNA sequences can explain many but not all of the hormone's actions [Norman, 1988].

Recently, a variety of cell types including osteoblasts [Lieberherr, 1987], osteoblastlike cells [Civitelli et al., 1990], intestine [Nemere et al., 1984], kidney [Suzuki et al., 1991], parathyroid cells [Boudreau et al., 1990], hematopoietic cells [Desai et al., 1986], muscle [Selles and Boland, 1991], chondrocytes [Schwartz et al., 1988], fibroblasts [Barsony and Marx, 1988], hepatocytes [Baran and Milne, 1986], keratinocytes [Smith and Holick, 1987], and insulinoma [Segrev and Rhoten, 1994] have been shown to respond rapidly (seconds to minutes) to 1α,25-$(OH)_2D_3$ with increases in intracellular calcium, pH, and cyclic nucleotides and alterations in phospholipid metabolism and protein kinase C activity. Indeed, other steroid

Contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

Grant sponsor: NIH; Grant number: DK39085.

*Correspondence to: Daniel T. Baran, M.D., Department of Orthopedics, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655.

Received 21 December 1999; Accepted 22 December 1999

This article published online in Wiley InterScience, April 2000.

hormones including estradiol [Aronica et al., 1994], testosterone [Lieberherr and Grosse, 1994], aldosterone [Wehling et al., 1994], and progesterone [Blackmore et al., 1991] all have been reported to exert effects by nongenomic mechanisms. For example, 1α,25-$(OH)_2D_3$, testosterone, estradiol, and progesterone have been shown to induce rapid transmembrane signaling pathways in rat osteoblasts [Lieberherr et al., 1994].

Although the rapid effects of 1α,25-$(OH)_2D_3$ on intracellular calcium, pH, and phospholipid metabolism suggested interaction with a rapid signaling system, it was unclear whether the functional nVDR was necessary for the hormone to exert its rapid effects. Using a rat osteoblast-like cell line that lacks the functional nVDR (ROS 24/1), we and others have demonstrated that 1α,25-$(OH)_2D_3$ rapidly increases intracellular calcium [Civitelli et al., 1990; Baran et al., 1991]. Nuclear extracts of these cells do not bind to the vitamin D response element (VDRE) of the osteocalcin (OC) gene [Baran et al., 1991]. In addition, the rapid signaling system has been shown to exhibit structural specificity for vitamin D analogs distinct from the nVDR [Baran et al., 1991; Farach-Carson et al., 1991; Norman et al., 1993a,b], suggesting that the rapid and genomic actions are mediated by different pathways.

We have used the 1α,25-$(OH)_2D_3$ analog, [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, to define membrane-associated proteins in ROS 24/1 cells, which specifically bind the hormone. The 1α,25-$(OH)_2D_3$ analog has been shown to produce rapid effects in ROS 24/1 cells [VanAuken et al., 1996]. It crosslinks covalently to proteins and allows for separation of the ligand–protein complex by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [Swamy and Ray, 1996; Swamy et al., 1998]. We have found that this analog binds specifically to a plasma membrane protein that was identified by sequence analysis and Western blot to be annexin II. In addition, 1α,25-$(OH)_2D_3$ exhibited specific and saturable binding to isoelectrically purified plasma membrane proteins approximating the pI of annexin II as well as to purified annexin II. Antibodies to annexin II diminished the binding of 1α,25-$(OH)_2D_3$ to the protein in partially purified plasma membranes, immunoprecipitated the hormone–ligand complex from plasma membrane preparations, and blocked the vitamin D–induced increases in intracellular calcium in ROS 24/1 cells. Taken together, these findings indicate that annexin II may serve as a receptor mediating the rapid effects of 1α,25-$(OH)_2D_3$ on intracellular calcium.

MATERIALS AND METHODS

Cell Cultures

Osteoblast-like rat osteosarcoma cells, ROS 24/1, were grown in culture medium consisting of Dulbecco's modified Eagle's medium and F12 (50:50) plus 10% fetal calf serum.

Cells were grown for 6–7 days and harvested for experiments by trypsinization with 0.25% trypsin and 0.002% ethylene-diaminetetraacetic acid and by sedimentation at 200*g* for 8 min. Cell numbers were assessed by counting an aliquot of cells in a hemocytometer, and viability was determined by trypan dye exclusion.

Isolation of Membranes

Partially purified plasma membranes were isolated as previously described [Baran et al., 1994]. ROS 24/1 cells were suspended in 20 mM Tris, pH 7.9, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ 10^8 cells/ml buffer and homogenized with 20 strokes in a tight-fitting Dounce homogenizer. The mixture was centrifuged for 8 min at 900*g* to remove debris and nuclei. The supernatant was centrifuged for 60 min at 100,000*g* to yield a crude membrane pellet. This preparation contained microsomes and mitochondria as well as the membranes but no intact cells or nuclei on light microscopy.

Plasma membrane proteins, pI 6.9–7.4, were purified by liquid phase isoelectric focusing using a Bio-Rad Rotofor system (Bio-Rad, Hercules, CA). The fractions were dialyzed to remove ampholytes before SDS-PAGE or binding studies.

Sequence Determination

The protein after electroblotting onto polyvinyl fluoride (PVDF) was digested in situ with trypsin in a digest buffer containing 100 mM ammonium bicarbonate, 10% acetonitrile, and 1% octylglucoside [Gharahdaghi et al., 1996]. A 0.5-μl aliquot of the digest was subjected to Matrix-Assisted Laser Desorption Time-of-Flight mass spectrometry (MALDI-TOF) using a Linear Biospectrometry Workstation (Preseptive Biosystems, Framingham, MA) and α-cyano-4-hydroxy cinnamic acid as the ma-

trix. The instrument was calibrated with an internal standard that consisted of angiotensin (MH+ of 1,297.5 Da) and ACTG (18–39; MH+ of 2,466.7 Da). Using just mass information, the NCBI nonredundant database was searched using the UCSF mass spectrometry facility's MSFIT program. The search was restricted to Rattus Norvegicus proteins in the 20,000–50,000-Da range using a 2-Da tolerance. Only one hit was observed, i.e., five of six masses matched annexin II. To further verify this identification, the digest mixture was separated on a 0.5-mm × 150-mm capillary C18 column (Applied Biosystems, Foster City, CA) that was coupled to an LC Packings (San Francisco, CA) flow splitter on a modified Hewlett Packard 1090 M HPLC system. Peptides were eluted using a linear gradient from 100% solvent A (0.1% trifluroacetic acid in water) to 46% solvent B (0.08% trifluroacetic acid in acetonitrile/water: 70/30) at a flow rate of 20 μl/min. The eluent was monitored at 210 nm, and fractions were collected manually. Selected fractions were analyzed by MALDI-TOF mass spectrometry, and a single mass fraction (1,478 Da) was selected for Edman sequence analysis using an Applied Biosystems 494 Procise protein sequencer. A single peptide sequence was observed (SYSPYDMLESIR), confirming the identification as annexin II.

Western Blot

The PVDF membrane with transferred protein was blocked with 5% nonfat dry milk dissolved in phosphate buffered saline and 0.1% Tween 20 (PBS-T) for 1 h at room temperature and then washed three times with PBS-T. The membrane was incubated at room temperature with annexin II mouse monoclonal antibody, 1/5,000 dilution in 1% bovine serum albumin (BSA)/PBS-T for 1 h, and washed five times before incubation with anti-mouse IgG: horseradish peroxidase, 1/7,500 dilution in 1% BSA/BS-T for 1 h. Annexin II protein was detected with NEN Chemiluminescence Reagent Plus (Boston, MA).

Binding Studies

Specific binding of [^{3}H]-1α,25-dihydroxyvitamin D_3 to isoelectrically purified plasma membrane proteins (20 μg protein) or annexin II (2 μg protein) was conducted in incubations containing 200 μl in a Tris buffer and either 20 μl of [^{3}H]-1α,25-dihydroxyvitamin D_3 (specific activity 165 mCi/mmole) or the [^{3}H] metabolite plus a 200-fold excess of unlabeled hormone. The incubations were performed overnight at 4°C. The bound and free metabolites were separated by addition of perchloric acid to a final concentration of 325 mM $HClO_4$ and 150 μg of bovine γ-globulin (Miles Scientific, Naperville, IL) for 30 min on ice [Nemere et al., 1994]. The precipitated proteins were pelleted in a microcentrifuge at top speed for 15 min in the cold. The pellets were solubilized in NaOH and CHAPS at 65°C before scintillation counting.

Immunoprecipitation

Plasma membrane proteins (100 μg) were labeled with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, 8,300 cpm, in 50 μl for 10 min. The membrane proteins were diluted to 250 μl and immunoprecipitated with annexin II mouse monoclonal antibody, 2 μg, for 18 h at 4°C before the addition of rabbit anti-mouse IgG, 5 μg, for 1 h at 4°C. The annexin II–antibody complex was adsorbed to 50% protein A sepharose for 2 h at 4°C, pelleted by centrifugation, resuspended in electrophoresis sample buffer, 100 μl, and boiled for 2 min. Proteins in the supernatant were precipitated with 4 volumes acetone, pelleted, redissolved in electrophoresis sample buffer, 100 μl, and boiled for 2 min. The proteins were electrophoresed on a 10% SDS-PAGE gel and transferred to an Immobilon PVDF membrane. The radiolabeled proteins were visualized by autoradiography.

Measurement of Intracellular Calcium

ROS 24/1 cells were cultured on glass coverslips and then incubated in HEPES/HCO_3 buffered saline at 37°C in the presence of fractionated immunoglobulins or anti–annexin II antibody for 1 h. Fura-2 acetoxymethyl ester (5 μM) was present for the final 20 min of incubation. The coverslips were then washed and mounted in a chamber. Each pseudocolor image was derived from a ratio pair of images produced by excitation at 340 nm and 380 nm light. Increases in intracellular calcium are indicated by a shift in color from blue toward red. Before initiation of the experiment, random cells were selected so that the fluorescence could be monitored at 10-s intervals. Intracellular calcium was calculated from the ratio of fluorescence (R) with excitation at 340 and 380

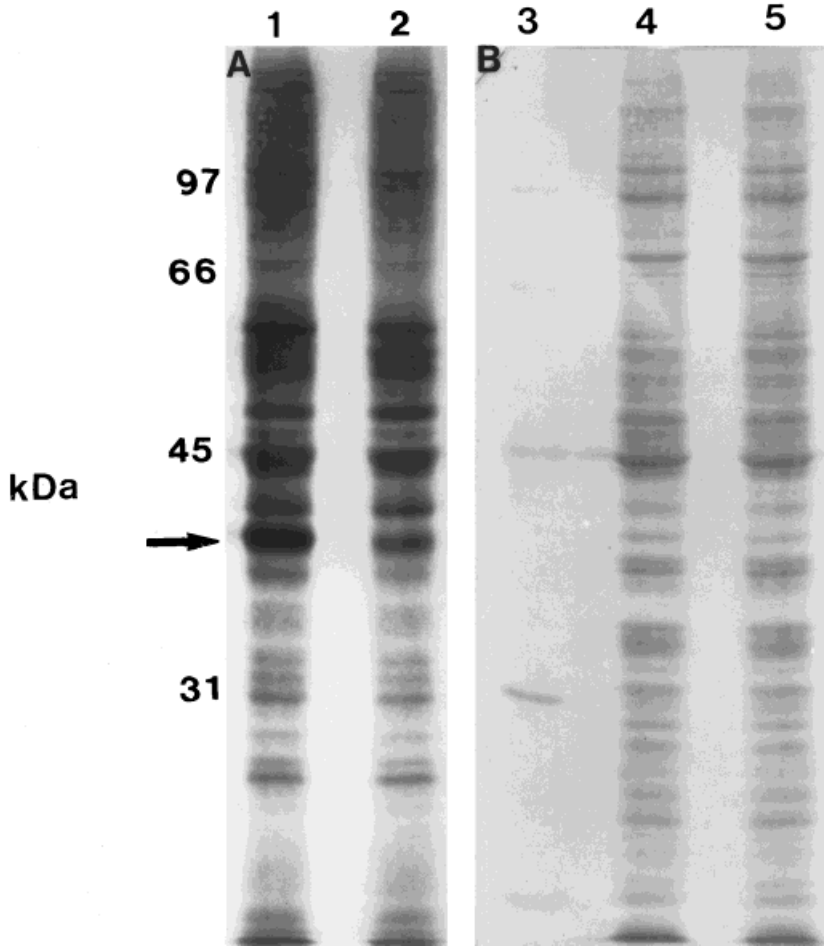

Fig. 1. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of partially purified plasma membranes. Partially purified plasma membranes, 330 μg (**lanes 1 and 2**), were treated with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (45,000 cpm, specific activity 10.9 mCi/mmole) in the absence (lane 1) or presence (lane 2) of 1α,25-dihydroxyvitamin D_3, 38 μM. The binding of the [^{14}C]-analog to a 36-kDa protein (arrow) is diminished by nonradioactive ligand, as shown in the autoradiograph (**A**, lane 2). **B** is the same membrane stained with amido black. Molecular weight markers (**lane 3**) are shown alongside plasma membranes (**lanes 4 and 5**).

nm according to the formula: $[\text{calcium}]_{\text{intracellular}} = K_d [(R - R_{min}/R_{max} - R)] \beta$, using an estimated K_d for Fura-2/calcium of 200 nM and R_{max} and R_{min} values obtained with Triton X-100 and EGTA, respectively.

Chemicals

Purified annexin II and polyclonal antibody to annexin II were purchased from Biodesign International (Kennebunk, ME). The monoclonal antibody to annexin II was obtained from Intransduction Laboratories (Los Angeles, CA). [^{3}H]-1α,25-dihydroxyvitamin D_3 was purchased from NEN Life Science Products (Boston, MA). 1α,25-Dihydroxyvitamin D_3 was generously supplied by Dr. Milan Uskovic (Hoffman LaRoche, Nutley, NJ), and the ROS 24/1 cells was supplied by Dr. Mark Haussler.

RESULTS

Partially purified plasma membranes from ROS 24/1 cells were incubated with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate in the absence and presence of nonradioactive 1α,25-$(OH)_2D_3$. A 36-kDa protein (Fig. 1, arrow) was specifically crosslinked to [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate in 12 separate experiments. Binding of the [^{14}C] analog to a 36-kDa protein was diminished by 1α,25-dihydroxyvitamin D_3, 38 μM, (Fig. 1) and by 10 μM and 3.3 μM cold ligand but not by 1 μM 1α,25-dihydroxyvitamin D_3 (data not shown). Internal sequence and mass spectroscopic data identified the major protein in this molecular weight band as annexin II. Purified annexin II and plasma membrane annexin II labeled with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate comigrated with immunoreactive annexin II, further confirming identity (Fig. 2). Anti-VDR antibodies did not recognize the labeled protein (data not shown).

Saturation analyses for [^{3}H]-1α,25-dihydroxyvitamin D_3 binding to isoelectrically purified plasma membrane proteins approximating the

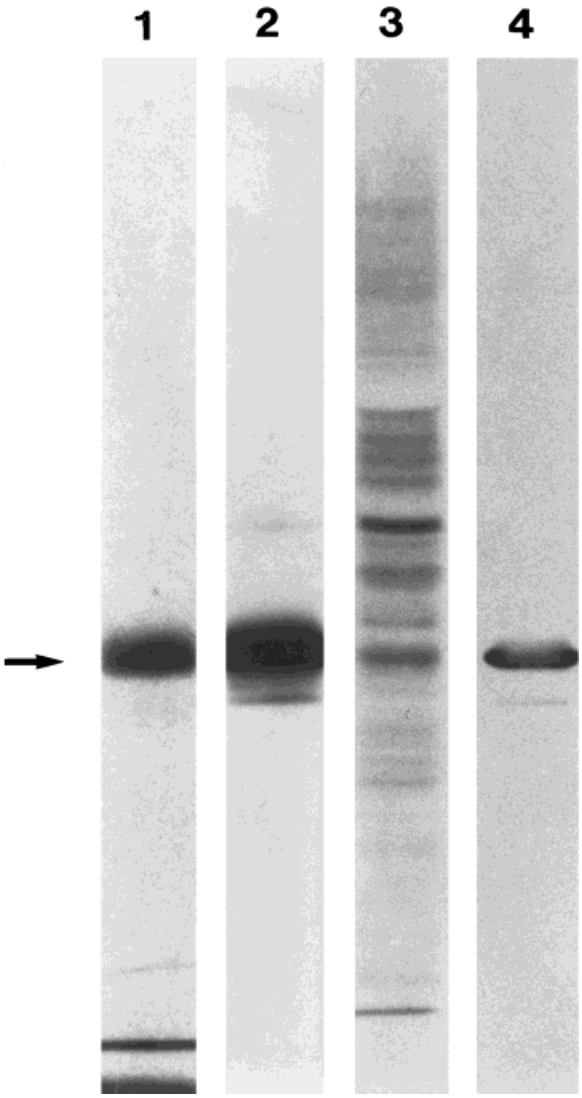

Fig. 2. Comigration of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate–labeled annexin II with immunoreactive annexin II. Purified annexin II, 1.5 μg (**lane 1**), and partially purified plasma membranes, 100 μg (**lane 3**), were treated with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (3,000 cpm, specific activity 10.9 mCi/mmole). The labeled annexin II (arrow) comigrated with immunoreactive annexin II (**lanes 2 and 4**) as detected by Western blot.

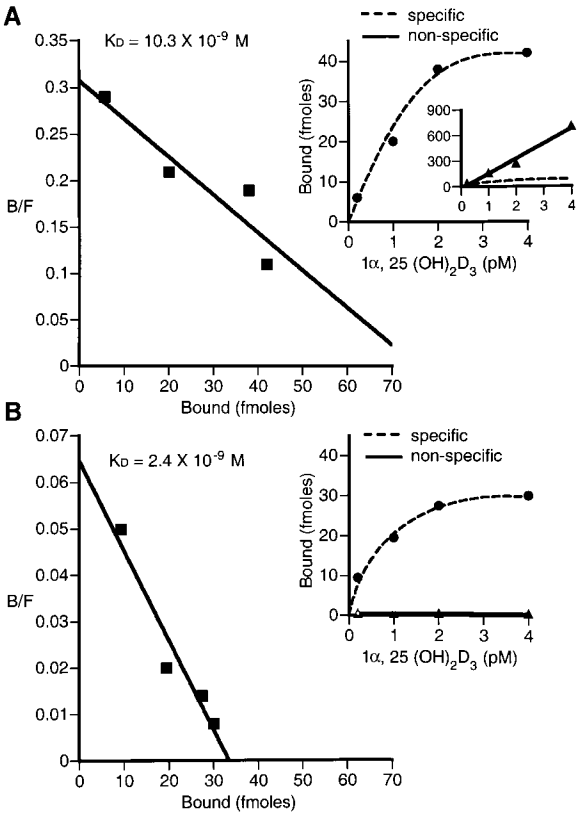

Fig. 3. 1α,25-Dihydroxyvitamin D_3 saturation analyses of isoelectrically purified plasma membrane proteins and annexin II. Solubilized plasma membrane proteins, pI 6.9–7.4 (20 μg protein), prepared by liquid phase isoelectric focusing (**A**) or purified annexin II (2 μg protein; **B**) were incubated with [^{3}H]-1α,25-dihydroxyvitamin D_3 (49,000 cpm, specific activity 165 Ci/mmole) in the absence or presence of a 200-fold excess of the hormone. Each point represents the mean of three observations. The dissociation constants were calculated from the best-fit linear equations. The results represent similar observations in two separate experiments. The smaller insert (A) shows specific and nonspecific binding of the bromoester to the isoelectrically purified membranes. The larger insert is drawn to the same scale as the insert in B, which shows specific binding to purified annexin II. There was no nonspecific binding to the purified protein.

pI of annexin II (pI 6.9–7.4) and purified annexin II were determined. The plasma membrane proteins were found to have a K_D of 10.3×10^{-9} M (Fig. 3A); the purified annexin II exhibited a K_D of 2.4×10^{-9} M (Fig. 3B). The B_{max} of the purified annexin II was 16 pmol/mg protein. Purified annexin II was crosslinked with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, and the protein separated under nondenaturing conditions. The [^{14}C]-1α,25-dihydroxyvitamin D_3 analog bound to proteins with molecular weights of approximately 36 kDa, 47 kDa, and 94 kDa, consistent with the monomeric, heterodimeric, and heterotetrameric forms, respectively, of annexin II (Fig. 4). It appears that all oligomeric forms of annexin II are capable of binding 1α,25-dihydroxyvitamin D_3, with the amount of binding being a function of the concentration of the oligomer.

The binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate to purified annexin II was greater than that to annexin I (Fig. 5). Excess

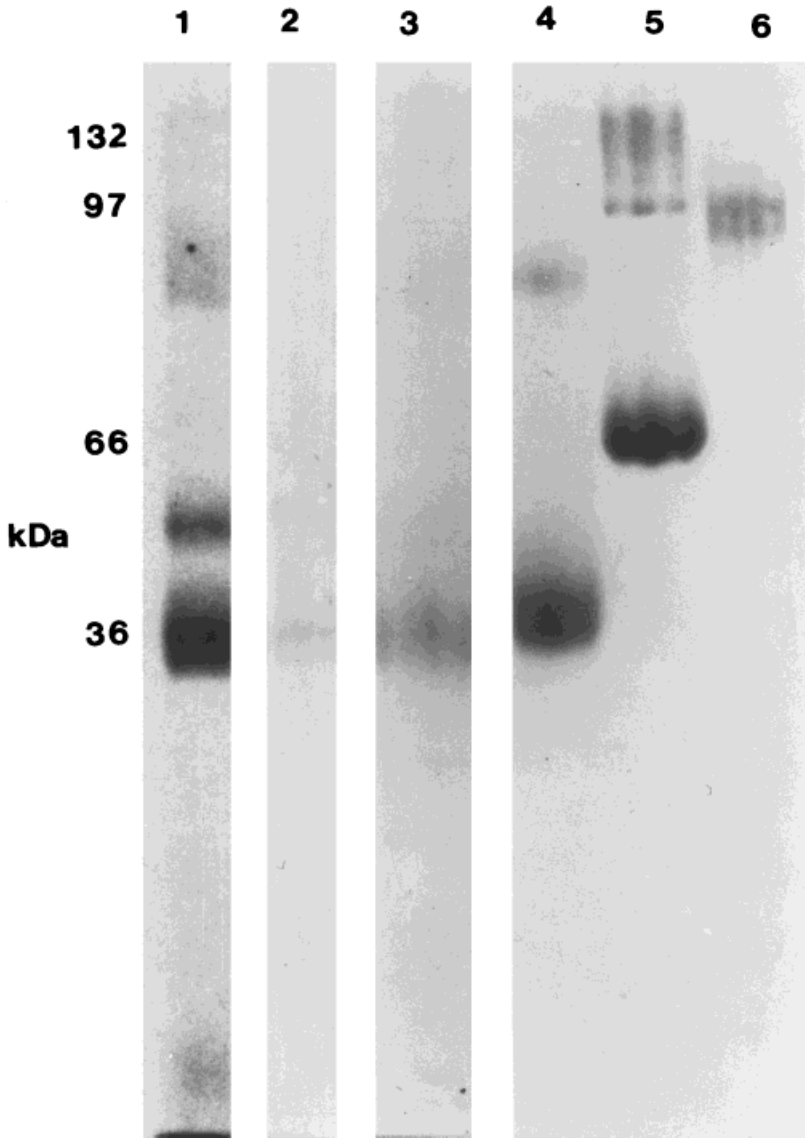

Fig. 4. Simultaneous determination of native and subunit molecular weights of annexin II. Annexin II, 4 μg, was crosslinked with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (3,000 cpm, specific activity 10.9 mCi/mmole) for 10 min before electrophoresis. The analog bound to three forms of annexin II that appear to correspond to the monomer, heterodimer, and heterotetramer (**lane 1**). Binding appeared to be a function of the concentration of the oligomer. The membrane was stained with amido black (**lane 2**), and the monomer and heterodimer forms were visualized. The heterotetramer could not be visualized. **Lanes 3–6** are molecular weight markers: lane 3, GAPDH; lane 4, ovalbumin; lane 5, bovine serum albumin; lane 6, phosphorylase β.

unlabeled 1α,25-dihydroxyvitamin D_3 markedly diminished the binding of the bromoacetate analog to purified annexin II (Fig. 5, lane 9) and minimally decreased binding to annexin I (Fig. 5, lane 7). Annexin I did not specifically bind [^{3}H]-1α,25-dihydroxyvitamin D_3 by saturation analysis (data not shown).

The binding of plasma membrane annexin II to the vitamin D bromoester analog was relatively specific because [^{14}C]-estradiol bromoacetate does not bind to annexin II (Fig. 6). On the contrary, the estradiol analog appeared to bind to many of the same proteins that bind [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate in a nonspecific manner. In addition, [^{3}H]-estradiol showed no specific binding to annexin II by Scatchard analysis (data not shown).

Partially purified plasma membranes from ROS 24/1 cells were preincubated with either polyclonal antibody to annexin II or fractionated immunoglobulins from pooled rabbit sera at 4°C for 2 h before treatment with the bromoester analog. The polyclonal antibody to annexin II decreased the binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate to the 36-kDa molecular-weight band (Fig. 7). The decrease in binding was quantitated by measuring radioactivity in the 36-kDa band. In four separate experiments, the average decrease in binding was 25%. The monoclonal

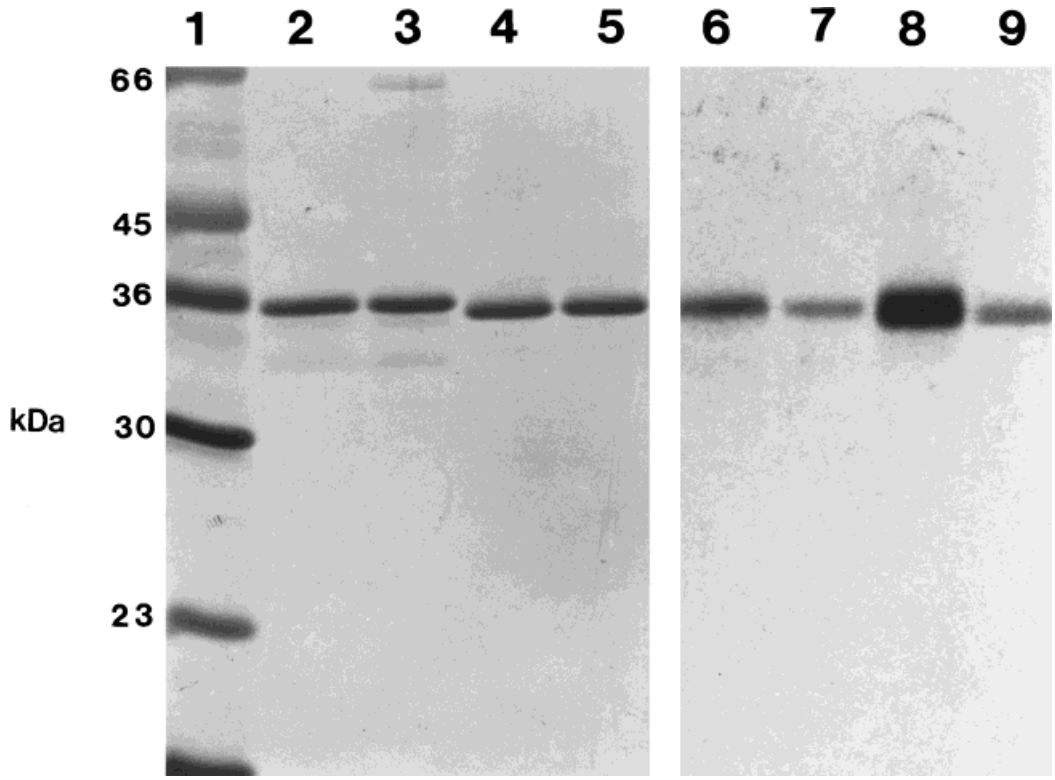

Fig. 5. Binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 to annexin I and II. The binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (2,000 cpm, specific activity 10.9 mCi/mmole) to annexin II, 1.5 μg (**lane 8**), is markedly diminished by excess 1α,25-dihydroxyvitamin D_3 19 μM (**lane 9**). The binding of the bromoester analog to annexin I, 1.5 μg (**lane 6**), is markedly diminished compared with annexin II (lane 8), whereas excess 1α,25-dihydroxyvitamin D_3 slightly decreases binding to annexin I (**lane 7**). Molecular weight markers are shown in **lane 1**, and **lanes 2–5** show the same membrane stained with amido black, demonstrating similar loading of the proteins.

antibody to annexin II was used for immunoprecipitation (Fig. 8). The antibody recognized the annexin II labeled with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (Fig. 8, lane 1). Western blot confirmed that the immunoprecipitated protein was annexin II (Fig. 8, lane 3).

To determine whether annexin II might be involved in the rapid actions of 1α,25-dihydroxyvitamin D_3 on intracellular calcium, ROS 24/1 cells were pretreated for 1 h with either the polyclonal annexin II antibody or the fractionated immunoglobulins. The cells were washed before stimulation with 1α,25-dihydroxyvitamin D_3. In cell populations pretreated with the control immunoglobulins, 1α,25-dihydroxyvitamin D_3, 20 nM, increased intracellular calcium (Fig. 9A). In cells pretreated with the polyclonal annexin II antibody, no response was noted (Fig. 9B). These results were observed in five separate experiments. Typical changes in intracellular calcium in an individual cell are shown in Figure 10. 1α,25-Dihydroxyvitamin D_3, 20 nM, increased intracellular calcium from 200 nM to 600 nM in cells pretreated with the control immunoglobulins (Fig. 10A). Increases in intracellular calcium in response to 1α,25-dihydroxyvitamin D_3 were never observed in cells pretreated with anti–annexin II antibody (Fig. 10B).

DISCUSSION

The annexins are a family of structurally related proteins that exhibit Ca^{2+}-dependent binding to phospholipids, in particular acidic phospholipids such as phosphatidylserine [for review, see Raynal and Pollard, 1994]. Annexin II, also known as lipocortin II and calpactin I heavy chain, can be found in vivo as either a 36-kDa monomer or as part of a heterodimer or heterotetramer comprised of one or two molecules of a 36-kDa monomer and one or two molecules of an 11-kDa protein, p11, respectively [Waisman, 1995]. The 36-kDa monomer is a peptide of 339 amino acids. The first 44

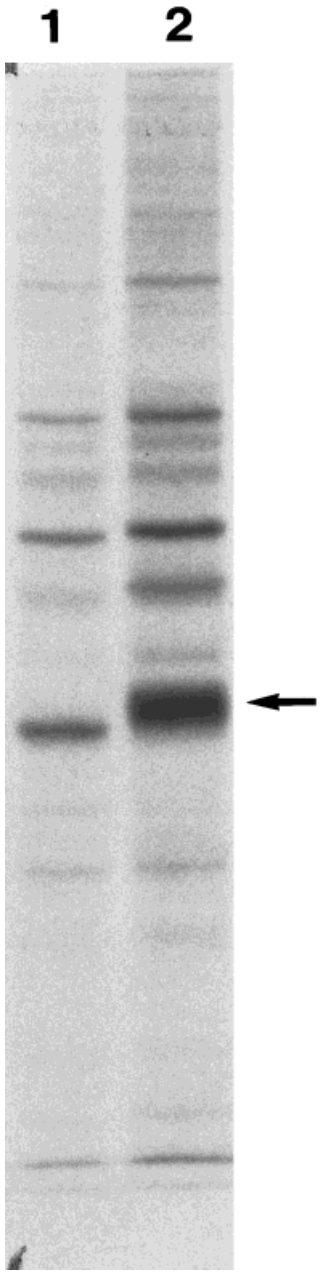

Fig. 6. Binding of [^{14}C]-estradiol bromoacetate and [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate to purified plasma membranes. [^{14}C]-Estradiol bromoacetate (3,000 cpm, specific activity 20 mCi/mmole) binds to many of the same membrane proteins (**lane 1**), as does [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate (3,000 cpm, specific activity 10.9 mCi/mmole; **lane 2**). The estradiol analog does not bind to annexin II (arrow).

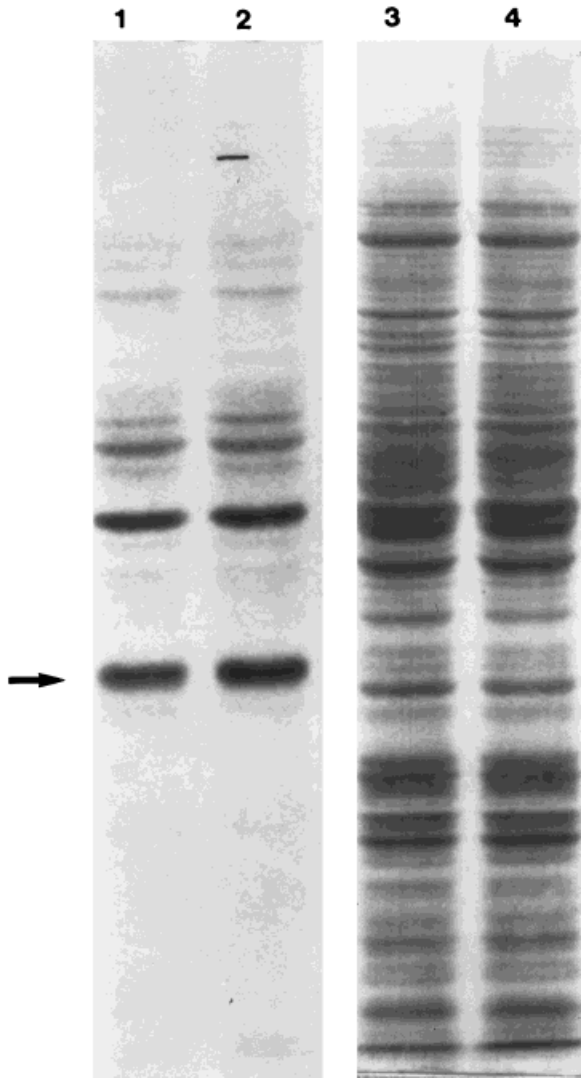

Fig. 7. Effect of anti–annexin II antibody on the binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate to plasma membrane annexin II. Partially purified plasma membranes, 120 μg protein, were treated with polyclonal anti–annexin II antibody (2 μg ammonium sulfate purified immune serum protein; **lane 1**) or 2 μg purified pooled rabbit protein serum (**lane 2**) for 2 h before exposure to [^{14}C]-1α,25-dihydroxyvitamin D_3-bromoacetate (12,000 cpm, specific activity 10.9 mCi/mmole). The binding of the [^{14}C] analog to annexin II (arrow) is diminished by the polyclonal anti–annexin II antibody (lane 1) as seen in the autoradiograph. **Lanes 3 and 4** show the same membrane stained with amido black.

N-terminal amino acids are unique to annexin II; the remainder of the annexin II molecule is composed of four core repeats that bind calcium and show 40–60% homology with the core repeats found in other annexin molecules. Human, bovine, and mouse annexin II are 98% identical [Raynal and Pollard 1994]. The function and significance of the three forms of annexin II (monomer, heterodimer, and heterotetramer) are unknown. The heterodimer (47 kDa) has been found in the nucleus and has been shown to regulate DNA polymerase α

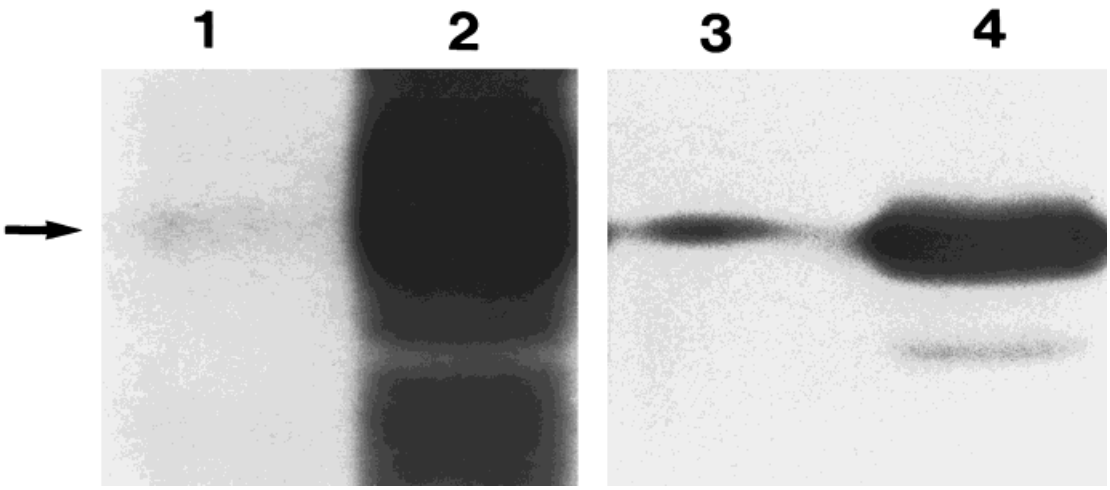

Fig. 8. Immunoprecipitation of annexin II. Plasma membrane proteins (100 μg) were labeled with [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate, 8300 cpm, for 10 min. Annexin II was immunoprecipitated with the mouse monoclonal antibody. The autoradiograph of the immunoprecipitated annexin II (**lane 1**) and the annexin II remaining in solution (**lane 2**) are shown on the left. The same membrane was used for Western blot (**lanes 3 and 4**).

[Jindal et al., 1991]. The heterotetramer (94 kDa) has been found in association with the plasma membrane [Thiel et al., 1992], and the monomer (36 kDa) has been identified in the cytosol and on plasma membrane inside and outside surfaces [Semich et al., 1989; Drust and Creaetz, 1991; Ma et al., 1994]. Both soluble and cytoskeletal forms of the annexin II monomer have been detected in human fibroblasts [Zokas and Glenney, 1987]. Annexin II monomer (36 kDa) present on the plasma membrane outside surface has been shown to be a receptor for tissue plasminogen activator [Cesarman et al., 1994; Hajjar et al., 1994] and for the alternatively spliced segment of tenascin-C [Chung and Erickson, 1994] and to possibly play a role in cell–cell interactions [Tessler et al., 1993]. Our observation that the polyclonal antibody to annexin II inhibits 1α,25-$(OH)_2D_3$-induced increments in intracellular calcium (Figs. 9, 10) supports the concept that annexin II can be located on the plasma membrane surface.

The present studies suggest that annexin II plays an essential role in mediating 1α,25-dihydroxyvitamin D_3 rapid signaling in osteoblasts. The annexins have previously been shown to be involved in bone cell metabolism. Annexins I, II, and VI are expressed by rat osteoblasts in primary culture [Suarez et al., 1993]. Annexin II has also been shown to modulate osteoclastic bone resorption. Annexin II, 5–1,000 ng/ml, significantly increased osteoclast multinucleated cell formation in human bone marrow cultures in the absence of 1α,25-dihydroxyvitamin D_3 and enhanced osteoclast multinucleated cell formation in mouse bone marrow cultures treated with 1α,25-dihydroxyvitamin D_3, 1 nM. The enhanced osteoclast formation in the murine cultures resulted in increased bone resorption as shown by an increased number of resorption lacunae formed on dentine slices. Treatment of fetal rat long bones with annexin II, 2 μg/ml, and 1α,25-dihydroxyvitamin D_3, 1 nM, significantly increased ^{45}Ca release, an index of bone resorption, as opposed to the hormone alone. Thus, annexin II appears to be involved in osteoclast multinucleated cell formation and the activity of these cells as regulated by 1α,25-dihydroxyvitamin D_3 [Takahashi et al., 1994]. Recently, annexin II has been shown to increase osteoclast formation. This affect appears to be mediated by annexin II activation of T cells, which in turn secrete GM-CSF, which expands the osteoclast precursor pool [Menaa et al., 1999].

Annexin II was the major protein present in the 36-kDa molecular-weight band that we sequenced (Fig. 1, arrow). It is possible that the [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate is specifically binding to another protein at this molecular weight, but one that is present in much smaller quantities. This is highly unlikely for several reasons. Annexin II binds the vitamin D bromoacetate analog (Figs. 4, 5), and

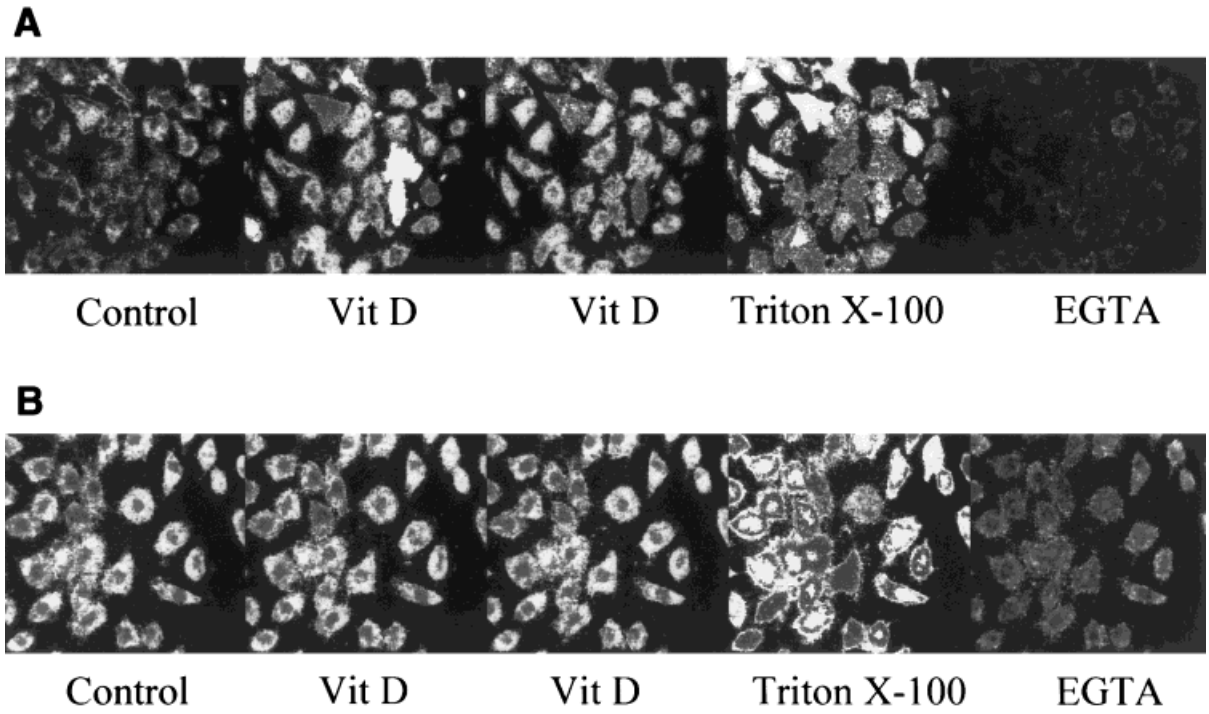

Fig. 9. Effect of anti–annexin II antibody on 1α,25-dihydroxyvitamin D_3–induced changes in intracellular calcium. Each panel includes sequential images taken before exposure to 1α,25-dihydroxyvitamin D_3 (Control), after two consecutive exposures to 20 nM, 1α,25-dihydroxyvitamin D_3 (Vit D), and after exposure to the nonionic detergent Triton X-100 (0.006%) in the absence and presence of the calcium chelator EGTA. The latter two conditions served to calibrate the fluorescent dye signal for high and low calcium, respectively. **A:** In cells pretreated with fractionated immunoglobulins, exposure to 1α,25-dihydroxyvitamin D_3 evoked an increase in calcium, but the response was not apparent in all cells. This is typical for vitamin D–induced changes in intracellular calcium. A second exposure to 1α,25-dihydroxyvitamin D_3 failed to produce additional changes. **B:** In cells exposed to anti–annexin II antibody, 1α,25-dihydroxyvitamin D_3 failed to increase intracellular calcium in any cell, although exposure to Triton X-100 and EGTA produced marked changes in fluorescence.

excess 1α,25-dihydroxyvitamin D_3 decreases the binding to purified annexin II (Fig. 5). The radioactivity-labeled annexin II, either the protein alone or in plasma membranes, comigrates with immunoreactive annexin II (Fig. 2). A monoclonal antibody that recognizes the carboxyterminal end of annexin II immunoprecipitates the protein labeled with the bromoester analog (Fig. 8). Finally, the saturation analyses of isoelectrically purified plasma membranes and annexin II are similar (Fig. 3), with no nonspecific binding detected to the pure protein (Fig. 3B).

The binding to purified annexin II is specific because [^{3}H]-estradiol does not bind to the protein. Similarly, [^{3}H]-1α,25-dihydroxyvitamin D_3 does not specifically bind to annexin I, a protein of similar molecular weight. The vitamin D bromoester bound to numerous other proteins in the membrane preparations (Figs. 1, 6). This may represent nonspecific binding of the steroid hormone or may be due in part to the bromoester moiety itself, which is responsible for the covalent binding.

Although the polyclonal antibody diminishes the binding of [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate to annexin II by only 25% in partially purified plasma membranes (Fig. 7), it completely inhibits the hormone-induced increases in intracellular calcium in intact cells (Figs. 9, 10). This observation suggests that the polyclonal antibody may be recognizing an epitope that is more critical for signal generation than for hormone binding. The rapid effects of 1α,25-dihydroxyvitamin D_3 in osteoblasts appear to involve alterations in phospholipid metabolism that mediate the subsequent increases in intracellular calcium [Civitelli et al., 1990]. Annexin II binds phospholipids, and this may play a key role in the membrane signaling pathway.

Despite using different antibody concentrations and detergent conditions to immunopre-

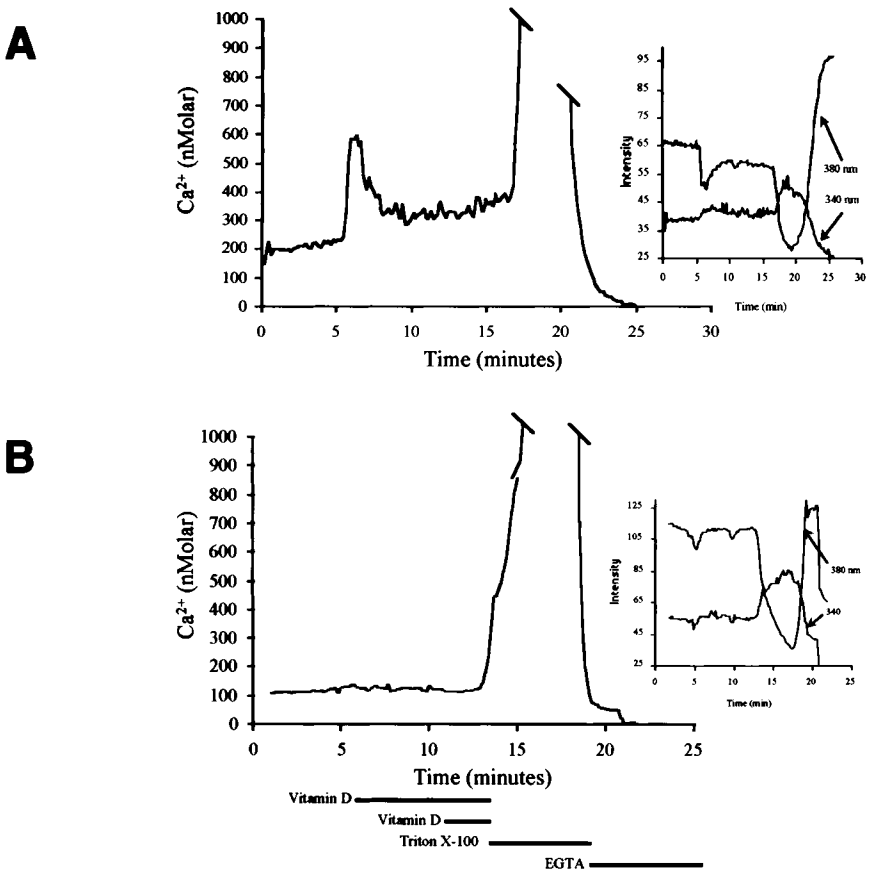

Fig. 10. Average changes in intracellular calcium. ROS 24/1 cells were incubated in the presence of fractionated immunoglobulins (**A**) or anti–annexin II antibody (**B**) as described in the caption to Figure 9. The inset in each panel shows the fluorescence signals observed at 340 and 380 nm used to calculate the ratio of fluorescence. In cells incubated with fractionated immunoglobulins, 1α,25-dihydroxyvitamin D_3 increased intracellular calcium threefold (average obtained from 10 cells). Subsequent exposure to additional 1α,25-dihydroxyvitamin D_3 failed to increase calcium further. Conversely, 1α,25-dihydroxyvitamin D_3 failed to affect intracellular calcium in cells incubated with anti–annexin II antibody (average obtained from 13 cells).

cipitate plasma membrane annexin II, the mouse monoclonal antibody to the protein precipitates only a small portion of the annexin II labeled with the vitamin D bromoester analog (Fig. 8). The polyclonal antibody to annexin II precipitated neither labeled annexin II nor immunoreactive annexin II. The relative amount of vitamin D–labeled annexin II that is precipitated parallels the amount of immunoreactive annexin II that is precipitated. The failure of antibodies to precipitate more of the annexin II is unexplained.

It has been reported by others that the membrane receptor for 1α,25-$(OH)_2D_3$ has a molecular weight between 60 and 66 kDa [Nemere et al., 1994, 1998]. A membrane fraction was used to generate polyclonal antibodies [Nemere et al., 1994]. One such antibody reacted with a 66-kDa protein in membranes of chondrocytes as detected by immunostaining and inhibited vitamin D–induced increments in protein kinase C activity [Nemere et al., 1998]. Demonstration of a protein by immunostaining with an antibody does not preclude binding of the antibody to another protein that cannot be detected by immunostain but is mediating the rapid actions. Although we also found that [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate bound to a triplet of proteins at molecular weight 66 kDa in partially purified plasma membrane of ROS 24/1 cells (Fig. 1, lane 1), binding was not diminished by nonradioactive ligand (Fig. 1, lane 2), suggesting that the binding to this particular protein is nonspecific.

Excess 1α,25-dihydroxyvitamin D_3 does appear to compete with the vitamin D bromoester for binding to other proteins. For example, in Figure 1, binding to proteins around 60 kDa and 34 kDa is diminished by unlabeled vitamin D. However, these observations were not consistent from experiment to experiment. The markedly diminished binding to the 36-kDa protein was always observed in the 12 experiments.

Estradiol [Aronica et al., 1994], testosterone [Lieberherr and Grosse, 1994], aldosterone [Wehling et al., 1994], and progesterone [Blackmore et al., 1991] have been reported to exert rapid nongenomic effects. To date, the putative membrane receptors that mediate these effects

have not been identified. It has been suggested that the rapid effects of estrogen are mediated by the nuclear estrogen receptor located in the plasma membrane [Razandi et al., 1999]. To examine the hormonal specificity of annexin II binding, we have compared [^{14}C]-estradiol bromoacetate and [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate–labeled plasma membranes (Fig. 6). Interestingly, although the estrogen analog binds to many of the same proteins that nonspecifically bind the vitamin D analog, the estrogen bromoester does not bind to annexin II, and specific binding of [^{2}H]-estradiol to annexin II cannot be demonstrated by saturation analysis.

Our present data suggest that annexin II may serve as a receptor in the transduction of the rapid actions of 1α,25-dihydroxyvitamin D_3. A protein that was specifically crosslinked to [^{14}C]-1α,25-dihydroxyvitamin D_3 bromoacetate was identified as annexin II by sequencing, comigration with immunoreactive protein, and immunoprecipitation. Specific and saturable binding of [^{3}H]-1α,25-dihydroxyvitamin D_3 to isoelectrically purified plasma membrane proteins and to purified annexin II was demonstrated with dissociation constants of 10.3×10^{-9} M and 2.4×10^{-9} M, respectively. [^{14}C]-1α,25-Dihydroxyvitamin D_3 bromoacetate bound to the 36-kDa monomer of annexin II and to the heterodimer and heterotetramer. This binding was diminished by excess 1α,25-dihydroxyvitamin D_3 and was much greater to annexin II than that to a protein of similar size and structure, annexin I. Binding of the bromoester analog to annexin II in partially purified plasma membranes was diminished by anti–annexin II polyclonal antibodies. The rapid effects of 1α,25-dihydroxyvitamin D_3, 20 nM, to increase intracellular calcium were not observed in cells pretreated with anti–annexin II antibodies. Identification of annexin II as the plasma membrane receptor for 1α,25-dihydroxyvitamin D_3 will allow the evaluation of the functional significance of the nongenomic actions of the hormone and provide a conceptual framework for evaluating the relationship of other members of the annexin family to the rapid actions of other steroid and steroidlike hormones.

REFERENCES

Aronica SM, Kraus WL, Katzenellenbogen BS. 1994. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci USA 91:8517–8521.

Baran DT, Milne ML. 1986. 1,25-Dihydroxyvitamin D_3 increases hepatocyte cytosolic calcium levels: a potential regulator of vitamin D-25-hydroxylase. J Clin Invest 77: 1622–1626.

Baran DT, Sorensen AM, Shalhoub V, Owen T, Oberdorf A, Stein G, Lian J. 1991. 1α,25-dihydroxyvitamin D_3 rapidly increases cytosolic calcium in clonal rat osteosarcoma cells lacking the vitamin D receptor. J Bone Min Res 6:1269–1275.

Baran DT, Ray R, Sorensen AM, Honeyman T, Holick MF. 1994. Binding characteristics of a membrane receptor that recognizes a 1α,25-dihydroxyvitamin D_3 and its epimer, 1β,25-dihydroxyvitamin D_3. J Cell Biochem 56: 510–517.

Barsony J, Marx SJ. 1988. Receptor mediated rapid action of 1α,25-dihydroxycholecalciferol: increase of intracellular cGMP in human skin fibroblasts. Proc Natl Acad Sci USA 85:1223–1226.

Blackmore PF, Neulen J, Lattanzio F, Beebe SJ. 1991. Cell surface binding sites for progesterone mediate calcium uptake in human sperm. J Biol Chem 266:18655–18659.

Boudreau A, Atmani F, Grosse B, Lieberherr M. 1990. Rapid effects of 1,25-dihydroxyvitamin D_3 and extracellular Ca^{2+} on phospholipid metabolism in dispersed porcine parathyroid cells. Endocrinology 127:2738–2743.

Cesarman GM, Guevara CA, Hajjar KA. 1994. An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). Annexin II mediated enhancement of t-PA dependent plasminogen activation. J Biol Chem 269: 21198–21203.

Chung CY, Erickson HP. 1994. Cell surface annexin II is a high affinity receptor for the alternatively spliced segment of tenascin-C. J Cell Biol 126:539–548.

Civitelli R, Kim YS, Gunstein SL, Fujimori A, Huskey M, Avioli LV, Hruska KA. 1990. Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology 127:2253–2262.

Desai SS, Appel MC, Baran DT. 1986. Differential effects of 1,25-dihydroxyvitamin D_3 on cytosolic calcium in two human cell lines (HL-60 and U-937). J Bone Min Res 1:497–501.

Drust DS, Creatz CE. 1991. Differential subcellular distribution of p36 (the heavy chain of calpactin I) and other annexins in the adrenal medulla. J Neurochem 56:469–478.

Farach-Carson MC, Sergeev I, Norman AW. 1991. Nongenomic actions of 1,25-dihydroxyvitamin D_3 in rat osteosarcoma cells: structure–function studies using ligand analogs. Endocrinology 129:1876–1884.

Gharahdaghi F, Kirchner M, Fernanadez J, Mische SM. 1996. Peptide mass profiles of polyvinylidene difluoride bound proteins by matrix assisted laser desorption/ionization time-of-flight mass spectrometry in the presence of nonionic detergents. Anal Biochem 233:94–99.

Hajjar KA, Jacovina AT, Chacko J. 1994. Endothelial cell receptor for plasminogen/tissue plasminogen activator: identify with annexin II. J Biol Chem 269:21191–21197.

Jindal HK, Chaney WG, Anderson CW, Davis RG, Vishwanatha JK. 1991. The protein tyrosine kinase substrate calpactin I heavy chain (p36) is part of the primer recognition protein complex that interacts with DNA polymerase α. J Biol Chem 266:5169–5176.

Lieberherr M. 1987. Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. J Biol Chem 262:13168–13173.

Lieberherr M, Grosse B. 1994. Androgens increase intracellular calcium concentration and inositol 1,4,5-triphosphate and diacylglycerol formation via a pertussis-sensitive G protein. J Biol Chem 269:7217–7223.

Lieberherr M, Grosse B, Tassin M-T, Kachkache M, Bourdeau A. 1994. Transmembrane signal pathways induced by calcitriol, estradiol testosterone, and progesterone in osteoblasts. Proceedings of the Ninth Workshop on Vitamin D, Orlando, Florida, May 28–June 2. pp 315–323.

Ma AS, Bell DJ, Mittal AA, Harrison HH. 1994. Immunocytochemical detection of extracellular annexin II in cultured human skin keratinocytes and isolation of annexin II isoforms enriched in the extracellular pool. J Cell Sci 107:1973–1984.

Menaa C, Devlin RD, Reddy SV, Gazitt Y, Choi SJ, Roodman GD. 1999. Annexin II increases osteoclast formation by stimulating the proliferation of osteoclast precursors in human marrow cultures. J Clin Invest 103:1605–1613.

Minghetti PP, Norman AW. 1988. 1α,25-$(OH)_2D_3$ Vitamin D_3 receptors: gene regulation and genetic circuitry. FASEB J 2:3043–3053.

Nemere I, Yoshimoto Y, Norman AW. 1984. Calcium transport in perfused duodena from normal chicks: enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D_3. Endocrinology 115:1476–1483.

Nemere I, Dormanen MC, Hammond MW, Okamura WH, Norma AW. 1994. Identification of a specific binding protein for 1α,25-dihydroxyvitamin D_3 in basal–lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 269:23750–23756.

Nemere I, Schwartz Z, Pedrozo H, Sylvia VL, Dean DD, Boyan BD. 1998. Identification of a membrane receptor for 1α,25-dihydroxyvitamin D_3 which mediates rapid activation of protein kinase C. J Bone Min Res 13:1353–1359.

Norman AW. 1988. Receptors for 1α,25-$(OH)_2D_3$: past, present, and future. J Bone Min Res 13:1360–1369.

Norman AW, Bouillo NR, Farach-Carson MC, Bishop JE, Zhou LX, Nemere I, Zhao J, Muralidharan KR, Okamura WH. 1993a. Demonstration that 1β,25-dihydroxyvitamin D_3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of 1α,25-dihydroxyvitamin D_3. J Biol Chem 268:20022–20030.

Norman AW, Okamura WH, Farach-Carson MC, Allewaert K, Branisteanu D, Nemers I, Muralidharan KR, Bouillon R. 1993b. Structure function studies of 1,25-dihydroxyvitamin D_3 and the vitamin D endocrine system. J Biol Chem 268:13811–13819.

Raynal P, Pollard HB. 1994. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium and phospholipid binding proteins. Biochim Biophys Acta 117:63–93.

Razandi M, Pedram A, Greene GL, Levin ER. 1999. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319.

Schwartz Z, Schlader DL, Swain LD, Boyan BD. 1988. Direct effects of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 on growth zone and resting zone chondrocyte membrane alkaline phosphatase and phospholipase A_2 specific activities. Endocrinology 123:2878–2884.

Segrev IN, Rhoten WB. 1994. Video imaging of intracellular calcium in insulinoma cells: effects of 1,25-$(OH)_2D_3$. Proceeding of the Ninth Workshop on Vitamin D, Orlando, Florida, May 28–June 2. pp 355–356.

Selles J, Boland R. 1991. Evidence on the participation of the 3′,5′-cyclic AMP pathway in the nongenomic action of 1,25-dihydroxyvitamin D_3 in cardiac muscle. Mol Cell Endocrinol 82:229–235.

Semich R, Gerke V, Robenek H, Weber K. 1989. The p36 substrate of $pp60^{src}$ kinase is located at the cytoplasmic surface of the plasma membrane of fibroblasts: an immunoelectron microscopic analysis. Eur J Cell Biol 50: 313–327.

Smith EL, Holick MF. 1987. The skin: the site of vitamin D_3 synthesis and a target tissue for its metabolite 1,25-dihydroxyvitamin D_3. Steroids 49:103–131.

Suarez F, Rothhut B, Comera C, Tongqui L, Marie FR, Silva C. 1993. Expression of annexin I, II, V, and VI by rat osteoblasts in primary culture: stimulation of annexin I expression by dexamethasone. J Bone Min Res 8:1201–1210.

Suzuki M, Kurihara S, Kawaguchi Y, Sakai O. 1991. Vitamin D_3 metabolites increase $[Ca^{2+}]_I$ in rabbit renal proximal straight tubule cells. Am J Physiol 260:F757–F763.

Swamy N, Ray R. 1996. Affinity labeling of the 1α,25-dihydroxyvitamin D_3 receptor. J Biol Chem 271:2012–2017.

Swamy N, Founine M, Ray R. 1998. Identification of the subdomain of the nuclear receptor for the hormonal forms of vitamin D_3, 1α,25-dihydroxyvitamin D_3, vitamin D receptor, that is covalently modified by an affinity labeling reagent. Arch Biochem Biophys 348:91–95.

Takahashi S, Reddy SV, Chirgwin JM, Devlin R, Anderson J, Roodman GD. 1994. Cloning and identification of annexin II as an autocrine/paracrine factor that increases osteoclast formation and bone resorption. J Biol Chem 269:28696–28701.

Tessler, RJ, Updyke TV, Yeatman T, Nicolson GL. 1993. Extracellular annexin II is associated with divalent cation dependent tumor cell–endothelia cell adhesion of metastatic RAW 117 large cell lymphoma cells. J Cell Biochem 53:265–276.

Thiel C, Osborn M, Gerke V. 1992. The tight association of the tyrosine kinase substrate annexin II with the submembranous cytoskeleton depends on intact p11 and Ca^{2+} binding sites. J Cell Sci 103:733–742.

VanAuken M, Buckley D, Ray R, Holick MF, Baran DT. 1996. Effects of the vitamin D_3 analog 1α,25-dihydroxyvitamin D_3-3β-bromoacetate on rat osteosarcoma cells: comparison with 1α,25-dihydroxyvitamin D_3. J Cell Biochem 63:302–310.

Waisman DM. 1995. Annexin II tetramer: structure and function. Mol Cell Biochem 149/150:301–322.

Wehling M, Ulsenheimer A, Schneider M, Neylon C, Christ M. 1994. Rapid effects of aldosterone on free intracellular calcium in vascular smooth muscle and endothelial cells: cellular localization of calcium elevation by single cell imaging. Biochem Biophys Res Commun 204:475–481.

Zokas L, Glenney JR. 1987. The calpactin light chain is tightly linked to the cytoskeletal form of calpactin I: studies using monoclonal antibodies. J Cell Biol 105: 2111–2121.