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Binding of 1α,25-Dihydroxyvitamin D_3 to Annexin II: Effect of Vitamin D Metabolites and Calcium

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Abstract We have recently reported that annexin II serves as a membrane receptor for 1α,25-$(OH)_2D_3$ and mediates the rapid effect of the hormone on intracellular calcium. The purpose of these studies was to characterize the binding of the hormone to annexin II, determine the specificity of binding, and assess the effect of calcium on binding. The binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to purified annexin II was inhibited by 1α,25-$(OH)_2D_3$ in a concentration-dependent manner. Binding of the radiolabeled ligand to annexin II was markedly diminished by 1α,25-$(OH)_2D_3$ at 24 μM, 18 μM, and 12 μM and blunted by 6 μM and 3 μM. At a concentration of 12 μM, 1β,25-$(OH)_2D_3$ also diminished the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II, but cholecalciferol, 25-$(OH)D_3$, and 24,25-$(OH)_2D_3$ did not. Saturation analyses of the binding of [^{3}H]-1α,25-$(OH)_2D_3$ to purified annexin II showed a K_D of 5.5×10^{-9} M, whereas [^{3}H]-1β,25-$(OH)_2D_3$ exhibited a K_D of 6.0×10^{-9} M. Calcium, which binds to the carboxy terminal domain of annexin II, had a concentration-dependent effect on [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate binding to annexin II, with 600 nM calcium being able to inhibit binding of the radiolabeled analog. The inhibitory effect of calcium was prevented by EDTA. Homocysteine, which binds to the amino terminal domain of annexin II, had no effect on the binding of the bromoacetate analog to the protein. The data indicate that 1α,25-$(OH)_2D_3$ binding to annexin II is specific and suggest that the binding site may be located on the carboxy terminal domain of the protein. The ability of 1β,25-$(OH)_2D_3$ to inhibit the binding of [^{14}C]-1α,25$(OH)_2D_3$ bromoacetate to annexin II provides a biochemical explanation for the ability of the 1β-epimer to inhibit the rapid actions of the hormone in vitro. J. Cell. Biochem. 80:259–265, 2000.

Key words: 1α,25-dihydroxyvitamin D_3; annexin II; rapid actions

1α,25-Dihydroxyvitamin D_3 has been shown to exert its effects in vitamin D-responsive cells by both genomic (minutes to hours) and rapid (seconds to minutes) mechanisms. We have recently reported that annexin II might serve as a membrane receptor for these rapid actions of 1α,25-$(OH)_2D_3$ [Baran et al., 2000]. 1α,25-$(OH)_2D_3$ was demonstrated to specifically bind to annexin II in plasma membranes as well as to purified annexin II. Antibodies to annexin II inhibited 1α,25-$(OH)_2D_3$ binding to annexin II, immunoprecipitated the ligand-protein complex, and inhibited 1α,25-$(OH)_2D_3$-induced increases in intracellular calcium in ROS 24/1 cells. The results indicated that annexin II serves as a membrane receptor for the rapid actions of 1α,25-$(OH)_2D_3$ on intracellular calcium.

The annexins are a family of structurally related proteins that exhibit Ca^{+2}-dependent binding to phospholipids [Raynal and Pollard, 1994]. Annexin II, also known as lipocortin II and calpactin I heavy chain, has been implicated in the regulation of a variety of activities both inside and outside the cell such as exocytosis, endocytosis, the structural organization of membranes, membrane-cytoskeleton interactions, Ca^{+2} homeostasis, the inflammatory response, and blood coagulation [Waisman, 1995]. Annexin II can be found in vivo as a 36-kDa monomer or as part of a heterodimer or heterotetramer composed of one or two mole-

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cule(s) of this 36-kDa monomer and one or two molecule(s) of an 11-kDa protein, p11. The 36-kDa peptide contains 339 amino acids: the first 44 aminoterminal amino acids are unique to annexin II. The remainder of the annexin II molecule is composed of four core repeats that show 40% to 60% homology with the core repeats found in other annexin molecules.

The amino terminal domain of annexin II containing serine and tyrosine phosphorylation sites [Raynal and Pollard, 1994], is a substrate for protein kinase C [Gould et al., 1986] and src [Cooper and Hunter, 1983], and is a binding site for homocysteine [Hajjar, 1993]. The carboxy terminal domain contains the sites for Ca^{+2} and phospholipid binding [Johnsson et al., 1986].

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 it has been shown to affect DNA polymerase α [Jindal et al., 1991]. The heterotetramer has been found in association with the plasma membrane [Thiel et al., 1992], and the monomer has been identified in the cytosol and on plasma membrane inside and outside surfaces [Drust and Creatz, 1991; Ma et al., 1994; Hajjar et al., 1996]. Both soluble and cytoskeletal forms of the annexin II monomer have been detected in human fibroblasts [Zokas and Glenney, 1987]. Annexin II is translocated to the outside cell surface within 16 h of its synthesis [Hajjar et al., 1996]. Cell-surface annexin II represents 4% to 5% of the total cell pool [Hajjar et al., 1996].

The purpose of these studies was to characterize the binding of 1α,25-$(OH)_2D_3$ to annexin II, determine the specificity of binding, and assess the effect of calcium and other cations on 1α,25-$(OH)_2D_3$ binding. The results indicate that 1β,25-$(OH)_2D_3$ competes with the hormone for binding to annexin II, whereas 24,25-$(OH)_2D_3$, 25(OH)D, and cholecalciferol do not. Calcium inhibits the binding of 1α,25-$(OH)_2D_3$ to annexin II, but homocysteine has no effect. The data suggest that 1α,25-$(OH)_2D_3$ binding to annexin II is specific and that the binding site may be located on the carboxy terminal domain of the protein.

MATERIALS AND METHODS

Binding Studies

Specific binding of [^{3}H]-1α,25-$(OH)_2D_3$ and [^{3}H]-1β,25-$(OH)_2D_3$ to annexin II (2 μg protein) was conducted in incubations containing 200μl in a Tris buffer and either 20 μl of [^{3}H]-1α,25-$(OH)_2D_3$ (specific activity 165 mCi/mmole) alone or with the addition of a 200-fold excess of unlabeled 1α,25-$(OH)_2D_3$, or 20 μl of [^{3}H]-1β,25-$(OH)_2D_3$ (specific activity 15 Ci/mmole) alone or with the addition of a 200-fold excess of unlabeled 1β,25-$(OH)_2D_3$. 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 for 15 min in the cold. The pellets were solubilized in NaOH and CHAPS at 65°C prior to scintillation counting.

Gel Electrophoresis and Western Blot

Annexin II was labeled with [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate, electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred to Immobilon PVDF membrane. The 1α,25-$(OH)_2D_3$ analog cross links covalently to proteins and allows for separation of the ligand-protein complex by SDS-PAGE [Ray et al., 1996; Swamy et al., 1998]. The radiolabeled proteins were visualized by autoradiography. The PVDF membrane with transferred protein was blocked with 5% nonfat dry milk dissolved in PBS-T (phosphate buffered saline-0.1% Tween 20) 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/PBS-T for 1 h and washed five times prior to incubation with horseradish peroxidase-labeled secondary antibody. Annexin II protein was detected with New England Nuclear (NEN) Chemiluminescence Reagent Plus.

Chemicals

Purified annexin II was purchased from Biodesign International, Kennebunk, ME. The monoclonal antibody to annexin II was obtained from Intransduction Laboratories, Los Angeles, CA. [^{3}H]-1β,25-$(OH)_2D_3$ and the unlabeled metabolite were synthesized in our laboratory [Ray and Holick, 1997]. [^{14}C]-1α,25-

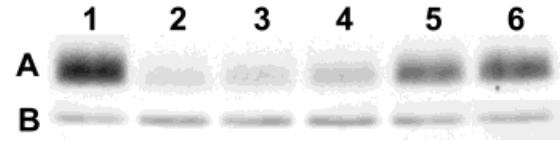

Fig. 1. 1α,25-$(OH)_2D_3$ inhibits [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate binding to annexin II. Annexin II (1.2 μg/100 μl) was incubated with vehicle (**lane 1**) or 1α,25-$(OH)_2D_3$ at 24 μM (**lane 2**), 18 μM (**lane 3**), 12 μM (**lane 4**), 6 μM (**lane 5**), and 3 μM (**lane 6**) for 60 min prior to addition of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate, 3,000 cpm, 0.6 μM for 10 min. The autoradiograph is shown in row A and the amido black stain of annexin II is shown in row B.

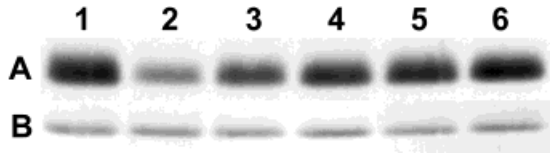

Fig. 2. Effect of vitamin D metabolites on [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate binding to annexin II. Annexin II (1.2 μg/100 μ1) was incubated with vehicle (**lane 1**), 1α,25-$(OH)_2D_3$ 12 μM (**lane 2**), 1β,25-$(OH)_2D_3$ 12 μM (**lane 3**), cholecalciferol 12 μM (**lane 4**), 25-(OH)D_3 12 μM (**lane 5**), or 24,25-$(OH)_2D_3$ 12 μM (**lane 6**) for 60 min prior to addition of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate, 3,000 cpm, 0.6 μM for 10 min. The autoradiograph is shown in row A and the amido black stain of annexin II is shown in row B.

$(OH)_2D_3$ was synthesized according to published procedures [Ray et al., 1996]. [^{3}H]-1α,25-$(OH)_2D_3$ was purchased from NEN Life Science Products, Boston MA. 1α,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, 25-(OH)D, and cholecalciferol were generously supplied by Dr. Milan Uskovic (Hoffman LaRoche, Nutley, NJ) and the ROS 24/1 cells were generously supplied by Dr. Mark Haussler. Homocysteine, calcium chloride, magnesium chloride, manganese chloride, and strontium chloride were purchased from Sigma, St. Louis MO.

RESULTS

The binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to purified annexin II was inhibited by 1α,25-$(OH)_2D_3$ in a concentration-dependent manner (Fig. 1). Binding of the radiolabeled ligand to annexin II was markedly diminished by 1α,25-$(OH)_2D_3$ at 24 μM, 18μM, and 12 μM; and it was blunted by 6 μM and 3 μM of the hormone. The effects of vitamin D metabolites on the binding of [^{14}C]-1α,25-$(OH)_2D_3$ to annexin II were evaluated using the unlabeled compounds at a concentration of 12 μM, the minimal concentration of 1α,25-$(OH)_2D_3$ that was maximally effective in blocking the binding of the bromoacetate analog (Fig. 1). 1α,25-$(OH)_2D_3$ diminished the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II, but cholecalciferol, 25-(OH)D_3, and 24,25-$(OH)_2D_3$ did not (Fig. 2). In contrast, 1β,25-$(OH)_2D_3$ also diminished binding of the bromoester to annexin II. Saturation analyses for [^{3}H]-1α,25-$(OH)_2D_3$ binding to isoelectrically purified plasma membrane proteins of ROS 24/1 cells approximating the pI of annexin II (pI 6.9–7.4) have previously been reported. The plasma membrane proteins were found to have a K_D of 10.3×10^{-9} M [Baran et al., 2000]. Saturation analyses of the binding of [^{3}H]-1α,25-$(OH)_2D_3$ to purified annexin II showed a K_D of 5.5×10^{-9} M, whereas 1β,25-$(OH)_2D_3$ had a K_D of 6.0×10^{-9} M (Fig. 3). Thus, it appears that 1β,25-$(OH)_2D_3$ is able to compete with [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate for binding to annexin II (Fig. 2) and binds to annexin II with a K_D similar to that of 1α,25-$(OH)_2D_3$ (Fig. 3).

Calcium, which binds to the carboxy terminal domain of annexin II, had a concentration-dependent effect on [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate binding to annexin II (Fig. 4) with 600 nM calcium being able to inhibit binding of the radiolabeled analog. The inhibitory effect of calcium on [^{14}C]-1α,25-$(OH)_2D_3$ binding to annexin II was prevented by ethylenediaminetetraacetate (EDTA; Fig. 5). Homocysteine, which binds to the amino terminal domain of annexin II, had no significant effect on the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to the protein (data not shown).

DISCUSSION

We have previously reported that annexin II may serve as a receptor for the rapid actions of 1α,25-$(OH)_2D_3$ [Baran et al., 2000]. A variety of cell types including osteoblasts [Lieberherr, 1987], osteoblast-like 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 rapidly respond (seconds to minutes) to 1α,25-$(OH)_2D_3$

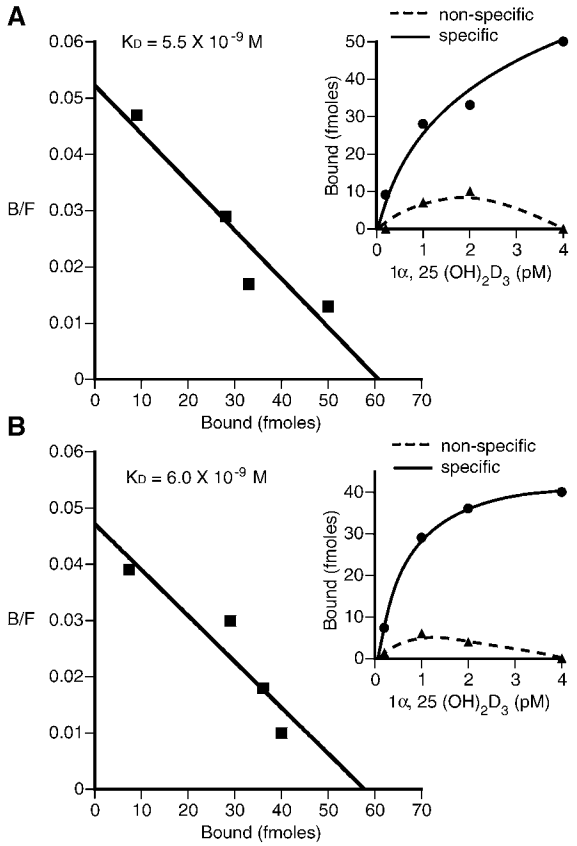

Fig. 3. Saturation analyses for [^{3}H]-1α,25-$(OH)_2D_3$ and [^{3}H]-1β,25-$(OH)_2D_3$ binding to annexin II. Purified annexin II (2 μg protein) were incubated with [^{3}H]-1α,25-$(OH)_2D_3$ (36,000 cpm, specific activity 165 Ci/mmole) in the absence or presence of 200-fold excess of the hormone (**A**) or with [^{3}H]-1β,25-$(OH)_2D_3$ (3,300 cpm, specific activity 15 Ci/mmole) in the absence or presence of 200-fold excess of the epimer (**B**). 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 inserts show specific and non-specific binding of the hormone and epimer to annexin II.

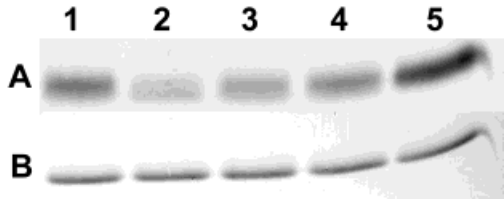

Fig. 4. Effect of calcium on [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate binding to annexin II. Annexin II (1.2 μg/100 μl) was incubated with 20 mM HEPES without calcium (**lanes 1 and 5**), HEPES with 2 mM calcium (**lane 2**), 100 μM calcium (**lane 3**), or 600 nM calcium (**lane 4**) for 20 min prior to the addition of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate (3,000 cpm, 0.6 μM) for 10 min. The autoradiograph is shown in row A and the amido black stain of annexin II is shown in row B.

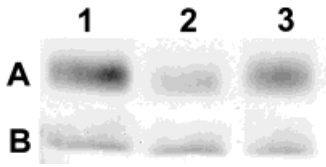

Fig. 5. Calcium inhibition of [^{14}C]-1α,25-$(OH)_2D_3$ bromoester binding to annexin II is reversed by EDTA. Annexin II (1.2 μg/100 μl) in 20 mM HEPES-buffered saline was incubated for 20 min in the absence or presence of $CaCl_2$, 2 mM, prior to the addition of EDTA, 5 mM, for 20 min. [^{14}C]-1α,25-$(OH)_2D_3$ bromoester (3,000 cpm) was added for 20 min prior to separation on a 10% SDS-PAGE gel. Calcium, 2 mM, decreased the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoester to annexin II (**lane 2**). The inhibitory effect of calcium was reversed by EDTA (**lane 3**). The autoradiograph is shown in row A and the amido black stain of annexin II is shown in row B.

with increases in intracellular calcium, pH, and cyclic nucleotides, and alterations in phospholipid metabolism and protein kinase C and src activity [Gniadecki, 1998; Revelli et al., 1998]. Studies suggest that other steroid hormones including estrogen [Buitrago et al., 2000], testosterone [Lieberherr and Grosse, 1994], aldosterone [Wehling et al., 1994], and progesterone [Burger et al., 1999] may also exert some of their effects by rapid nongenomic actions [Wehling, 1994]. Furthermore, like 1α,25-$(OH)_2D_3$, testosterone [Lieberherr and Grosse, 1994], estradiol [Lieberherr et al., 1994], and progesterone [Lieberherr et al., 1994] have been shown to induce transmembrane signaling pathways in rat osteoblasts. The ability of 1α,25-$(OH)_2D_3$ and a number of other steroid hormones to exert rapid effects on a variety of tissues suggests that these actions are functionally significant and are not limited to one steroid.

Specific steroid binding to plasma membranes has been noted for aldosterone [Christ et al., 1994], corticosterone [Orchinik et al., 1991], dexamethasone [Quelle et al., 1988], estradiol [Pappas et al., 1995; Bression et al., 1986], progesterone [Ke and Ramirez, 1990], and pregnenolone sulfate [Majewska et al., 1990]; however, specific protein "receptors" for these hormones have not been identified. As a result of studies in which the nuclear estrogen receptor is overexpressed, it has been suggested that the nuclear estrogen receptor may also mediate the rapid actions of estrogen [Razandi et al., 1999]. Conversely, the rapid effects of aldosterone persist in mineralocorti-

coid receptor knockout mice, suggesting the nongenomic effects of the mineralocorticoid are mediated by a separate signaling system [Haseroth et al., 1999]. We have shown that the rapid effects of 1α,25-$(OH)_2D_3$ on intracellular calcium in ROS 24/1 cells are prevented by antibodies to annexin II [Baran et al., 2000]. Hence, it appears that the rapid effects of aldosterone, like those of 1α,25-$(OH)_2D_3$, are mediated by a signaling system distinct from the nuclear receptor.

Alterations in the level of expression of annexin in humans have pathologic consequences, and the term annexinopathies has been used to describe this class of diseases [Rand, 1999]. The annexin II monomer (36 kDa), present on the plasma membrane outside surface, has been shown to bind tissue plasminogen activator [Cesarman et al., 1994; Hajjar et al., 1994]. High levels of annexin II in humans are associated with fibrinolysis, which is inhibited by polyclonal anti-annexin II antibodies [Menell et al., 1999]. It has been suggested that enhanced tissue plasminogen binding to annexin II due to increased levels of annexin mediates the fibrinolysis. In contrast, homocysteine, which binds to the amino terminal domain of annexin II, inhibits the binding of tissue plasminogen activator to the protein [Hajjar, 1993]. Hence, homocysteine inhibition of tissue plasminogen activator binding to annexin II may explain the prothrombotic state associated with homocystinuria [Hajjar and Jacovina, 1998]. Homocysteine does not appear to significantly affect the labeling of annexin II by [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate.

Calcium inhibits the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II in a concentration-dependent manner (Fig. 4). Calcium has been reported to bind to the carboxy terminal domain of annexin II, induce a large conformational change in the molecule, and influence the spectral properties of the single tryptophan and one tyrosine [Johnsson et al., 1986]. The ability of the cation to inhibit the binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II, and the lack of an effect of homocysteine on binding, suggest that the hormone binds to the carboxy terminal domain of annexin II.

1β,25-$(OH)_2D_3$ competes with 1α,25-$(OH)_2D_3$ for binding to annexin II (Fig. 2). The K_D of both metabolites for annexin II is similar (Fig. 3). The 1β epimer of 1α,25-$(OH)_2D_3$, which does not interact with the nuclear vitamin D receptor [Holick et al., 1980], inhibits the rapid effects of the hormone [Baran et al., 1991; Norman et al., 1992; Norman et al., 1993; Zanello and Norman, 1997]. This suggested that the epimer bound to but did not activate the signaling system mediating the rapid actions of 1α,25-$(OH)_2D_3$ [Baran et al., 1991]. Our present studies demonstrate that the 1β epimer competes with the hormone for binding to annexin II. This may explain the ability of the epimer to inhibit the rapid actions of vitamin D.

It is not surprising that the concentrations of 1α,25-$(OH)_2D_3$ and 1β,25-$(OH)_2D_3$ required to inhibit binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II (Fig. 2) are greater than the concentrations needed to inhibit binding of [^{3}H]-1α,25-$(OH)_2D_3$ to annexin II in the saturation analyses (Fig. 3). High concentrations of the hormone and its 1β epimer were required to diminish the binding of the bromoester analog to annexin II. These studies do not indicate the affinity of the hormone or the epimer for annexin II, since the bromoester analog binds covalently and once bound cannot be displaced [Swamy et al., 1998]. This covalent cross-linking allows for separation of the ligand-protein complex by SDS-PAGE (Fig. 2).

In summary, we have shown that specific binding of [^{14}C]-1α,25-$(OH)_2D_3$ bromoacetate to annexin II is inhibited by the 1β epimer of the hormone and calcium. The data suggest that 1α,25-$(OH)_2D_3$ binds to the carboxy terminal domain of annexin II and provide a biochemical explanation for the ability of the 1β epimer to inhibit the rapid actions of the hormone in vitro.

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