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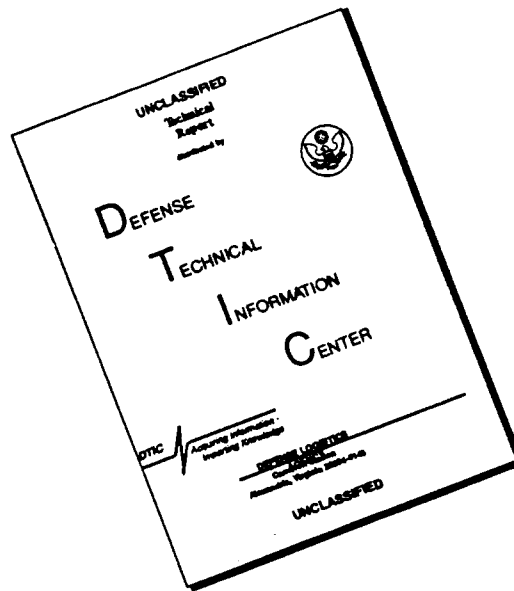
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13. ABSTRACT (Maximum 200) <p style="text-align: justify;">Tamoxifen is a major therapeutic agent in the treatment of breast cancer. While tamoxifen acts as an estrogen antagonist in breast tissue, its actions on other tissues reflect a range of estrogenic and antiestrogenic activity. Both estrogen and tamoxifen act to preserve bone mass, but the mechanisms underlying this effect are not fully understood. The goal of this study was to determine the effects of estrogen and tamoxifen on protein kinase C (PKC) isozyme expression in osteoblasts; this signaling pathway is estrogen-responsive in other tissues. We have established the PKC isozyme profile in a number of normal and tumor-derived osteoblast cell lines. The characteristic isozymes present in these cells are PKC-α, β_1, ϵ, ζ, and ι. The time course and dose dependence of their regulation by the phorbol ester phorbol 12, 13-dibutyrate was also defined. A number of different treatment protocols for estrogen and tamoxifen were employed. Both 72 h and 7 day treatment of subconfluent UMR-106 cells with tamoxifen increased the expression of PKC-β_1; additional experiments are necessary to further characterize these effects. Future directions of this research will explore the role of this isozyme in the actions of tamoxifen on bone.</p>			
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Jennifer L. Sanders 1/22/96
PI - Signature Date

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INTRODUCTION

The synthetic antiestrogen tamoxifen (Nolvadex) is a major therapeutic agent in the treatment of breast cancer. The usefulness of tamoxifen in treating this disease derives from the role that ovarian hormones play in the growth and maintenance of breast tumors⁽¹⁾; approximately 30% of breast cancers are hormone-responsive, and some breast tumors are believed to be directly dependent on estrogen for growth. Currently tamoxifen is used in over 80 countries to treat breast cancer, most commonly as an adjunct to surgery in the treatment of primary breast cancer⁽²⁾. In addition, tamoxifen has been shown to be effective in preventing breast cancer in both experimental and clinical studies⁽³⁻⁷⁾. Further studies are in progress to explore the efficacy of this agent for the prevention of breast cancer.

Although the antiestrogenic action of tamoxifen is well established in breast tissue, its effects in other human tissues are not fully understood. By definition, antiestrogens block the actions of estrogens. However, most known antiestrogens, including tamoxifen, also have agonistic properties which are typically both species and tissue specific⁽⁸⁾. Since long courses of tamoxifen therapy are currently favored⁽⁹⁾ and because this drug may be given for extended periods of time in the future to prevent breast cancer, it is important to fully understand the effects of tamoxifen on other estrogen-sensitive tissues and organs.

One important target tissue for estrogen is bone. Estrogen is important in maintaining bone mass in premenopausal women, and hormone replacement therapy is widely employed after menopause to prevent osteoporosis. Based on its antiestrogenic character, it was suggested that tamoxifen could promote osteoporosis. Several experimental and clinical studies, however, demonstrated that tamoxifen has estrogenic effects on bone thereby preserving bone mass⁽¹⁰⁻¹³⁾.

What role do estrogen and tamoxifen play in preserving bone mass? Both agents preserve mass by influencing bone remodeling. Bone remodeling is a process involving the removal of old bone (resorption) and its replacement with new bone. Bone resorption and formation are mediated primarily by two bone cell types; osteoclasts resorb bone and osteoblasts form new bone. Hormones, such as estrogen, and local factors, including growth factors, cytokines, and prostaglandins, act to regulate the balance between remodeling activities^(14,15). Estrogen and tamoxifen act primarily as anti-resorptive agents in bone, therefore it is reasonable to assume that they act directly on osteoclasts. The hormonal regulation of bone resorption by osteoclasts occurs by an osteoblast-mediated mechanism⁽¹⁶⁾, however, establishing the importance of the osteoblast in both aspects of bone remodeling.

Though the role of estrogen and tamoxifen as anti-resorptive agents is fairly clear, the molecular mechanisms through which these agents exert their effects on bone are not well understood. It is well established that nuclear receptors for estrogen are present in osteoblastic cells^(17,18), and there is limited evidence from avian tissues for the presence of estrogen receptors in osteoclasts⁽¹⁹⁾. Estrogen-receptor complexes are known to interact with specific genes and regulate their transcriptional activity⁽²⁰⁾. Similarly, tamoxifen-estrogen receptor complexes bind specifically to estrogen-responsive elements, apparently to the same sites as estradiol-estrogen receptor complexes⁽²¹⁾. In select tissues, including rat pituitary⁽²²⁾ and rabbit⁽²³⁾ and rat⁽²⁴⁾ ovary, estrogen has been shown to modulate protein kinase C (PKC) expression. PKC is involved in the proliferation and differentiation of many cell types⁽²⁵⁾ and is a major signalling pathway in bone

cells⁽²⁶⁻³⁵⁾. Based on this information, a link between estrogen/tamoxifen and PKC expression in bone is plausible as well.

The PKC pathway in bone, as in other tissues, is more complex than initially realized. PKC is not a single enzyme, but a family of serine/threonine kinases. The PKC family consists of at least 11 isozymes with unique tissue distributions and substrate specificities⁽³⁶⁻⁴³⁾. These isozymes comprise at least three classes, termed the conventional, novel, and atypical isozymes. The PKC isozymes in a given class have similar activation requirements due to common structural features within each class (Figure 1, see Appendix 1). The conventional isozymes, PKC- α , - β _I, - β _{II}, and - γ , require phosphatidylserine (PS), diacylglycerol (DAG), and calcium (Ca^{2+}) for activation⁽³⁶⁻³⁸⁾. The novel isozymes, PKC- δ , - ϵ , - η , and - θ also require PS and DAG for activation, but are Ca^{2+} -independent⁽³⁶⁻⁴¹⁾. The atypical isozymes, PKC- ζ and - ι/λ , require PS but are both Ca^{2+} - and DAG-independent^(36-38,42,43).

The goal of the studies described here was to determine whether estrogen and tamoxifen modulate PKC isozyme expression in osteoblasts. Due to the presence of multiple isozymes of PKC, such studies should be a good discriminator of the similarities and differences of the actions of estrogen and tamoxifen on bone. Because little is known about specific PKC isozymes in bone tissue, we first characterized the expression and phorbol ester-induced down-regulation of PKC isozymes in osteoblastic cells. This characterization was achieved through Western immunoblotting with isozyme-specific anti-PKC antibodies. Western blotting was also employed to examine isozyme expression following treatment of UMR-106 rat osteoblastic osteosarcoma cells with 17β -estradiol and tamoxifen. Since the effects of estrogen/tamoxifen on isozyme expression have not been previously studied in bone, we explored a range of hormone treatment protocols.

BODY

Experimental Methods

Cell Culture

Normal mouse osteoblasts were isolated enzymatically under sterile conditions from neonatal (5-6 day) mouse calvaria using a method described previously⁽⁴⁴⁾. This method was modified, however, to include six 20 min incubations with collagenase. After isolation, osteoblasts were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated horse serum, 100 U/ml K-penicillin G, and 10 U/ml Na-heparin, and added to culture dishes. The cells were allowed to attach overnight at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed the following day to remove unattached cells.

UMR-106 rat osteoblastic osteosarcoma cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in 75 cm² cell culture flasks at 37°C in a humidified 5% CO₂ atmosphere in DMEM supplemented with 15% heat-inactivated horse serum and 100 U/ml K-penicillin G. UMR-106 cells between the 4th and 17th passage were used for experiments. The ROS 17/2.8 and ROS 24/1 rat osteoblastic cell lines were provided by Dr. S.B. Rodan from Merck and were cultured as previously described⁽⁴⁵⁾. Rat cells were passaged every 5-7 days with medium changes every 3 days. The four human osteoblastic cell lines used, MG-63, G-292, SaOS-2, and HOS-TE85, (American Type Culture Collection) were cultured under conditions recommended by the supplier.

Experimental protocols

A. Isozyme expression in osteoblastic cells

The normal osteoblasts and the osteoblastic cell lines were approximately 40-50% confluent when harvested.

B. Determination of the sensitivity of PKC isozymes to prolonged phorbol ester treatment

Normal mouse osteoblasts and UMR-106 rat osteoblastic cells were selected for phorbol ester time course studies. After isolation, normal mouse osteoblasts were seeded in 10 cm dishes in 10 ml of culture medium. The medium was changed on both the first and second days after isolation. On the third day after isolation, treatments were initiated. Phorbol 12, 13-dibutyrate (PDB; Sigma Chemical Company, St. Louis, MO, U.S.A.) or an equal volume of vehicle (dimethyl sulfoxide (DMSO); Sigma) was added directly to the culture medium to give a final PDB concentration of 1 µM; the contents of the dishes were mixed by swirling. Cells were treated with PDB or vehicle for 1, 3, 6, 12, 24, or 48 h.

Time course experiments with the UMR-106 cells were carried out as described for the normal mouse osteoblasts except that phorbol ester or vehicle treatments were initiated the day after the UMR-106 cells were seeded (1.5 x 10⁶ cells/dish) into 10 cm dishes.

Dose response and withdrawal experiments with PDB were carried out with UMR-106 cells, as were experiments with the inactive phorbol, 4 α -phorbol 12, 13-didecanoate (α -PDD; Sigma). For each of these studies, UMR-106 cells were seeded in 10 cm dishes (1.5×10^6 cells/dish), and treatments initiated the following day. For dose response studies, cells were treated for 24 or 48 h with vehicle or 0.3 μ M, 1 μ M, or 3 μ M PDB. Withdrawal experiments involved 3 dishes of cells; one dish was treated with vehicle for 48 h, the second with 1 μ M PDB for 48 h, and the third with 1 μ M PDB for the first 24 h and vehicle for the final 24 h. After the initial 24 h period, all dishes were handled in the same way; that is, the medium was aspirated, the cells were washed 3X with culture medium, and fresh treatments were added for an additional 24 h. For the α -PDD studies, cells were treated with either vehicle or 1 μ M α -PDD for 48 h.

C. Hormone treatment

24 h after the osteoblastic cells were plated, the medium was changed to phenol red-free medium with 15% charcoal-stripped horse serum (UMR-106 cells) or 5% charcoal-stripped fetal bovine serum and 5% charcoal-stripped horse serum (MG-63 cells) to reduce the effects associated with endogenous estrogens. 48 h after plating, confluent cells were treated with vehicle (absolute ethanol) or hormone (10^{-12} to 10^{-7} M; 17 β -estradiol, 4-hydroxy-tamoxifen (4-OH-tamoxifen)). Cells were incubated with vehicle/hormone for 24 h before harvesting.

Additional treatment strategies employed in this study will be described in the Results section.

Western immunoblotting

The isozyme-specific anti-PKC antibodies utilized for Western blotting (rabbit polyclonal antibodies raised against C-terminal isozyme peptides) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Cells were harvested for Western blotting according to the method suggested in the research applications supplement received with the antibodies; this method is described below.

Cells were removed from culture dishes by scraping in RIPA buffer (phosphate buffered saline (PBS), 1% Nonidet-P40 (NP40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.15-0.3 TIU/ml aprotinin, 1 mM sodium orthovanadate). The cell suspension was transferred to a 1.5 ml microfuge tube using a syringe fitted with a 21 gauge needle. The dish was washed once with additional RIPA buffer, and this was combined with the first lysate. The total volume of lysate was then passed through a 21 gauge needle approximately 15X to shear the DNA. After a 30 min incubation on ice, additional PMSF (0.1 mg/ml) was added to each lysate. The samples were microfuged for 20 min at 4 $^{\circ}$ C, and the resulting supernatant was taken as the total cell lysate. An aliquot of the supernatant was removed for determination of protein, which was measured by the method of Lowry⁽⁴⁶⁾. The remaining supernatant was mixed with an equal volume of stop solution (17% glycerol, 8.7% (v/v) 2-mercaptoethanol, 5% SDS, 0.2 M Tris-HCl (pH 6.7), 0.1 mg/ml bromophenol blue) and boiled for 2 min. Samples were stored at -20 $^{\circ}$ C until use.

For immunoblotting⁽⁴⁷⁾, extracts were subjected to SDS polyacrylamide electrophoresis using 5% stacking gels and 10% separating gels (0.05 amp/gel, 4-5 h) followed by electrophoretic

transfer of proteins to nitrocellulose (0.2 μ M, Schleicher & Schuell, Keene, NH, U.S.A.) (30 volts, overnight). After transfer, membranes were blocked in Blotto B (Tris-buffered saline (TBS), 0.05% Tween-20 (T), 1% bovine serum albumin (BSA), 1% Carnation non-fat dry milk) for 1 h at room temperature. Blots were then incubated with isozyme-specific anti-PKC antibodies (1:1000 in Blotto B) for 45 min at room temperature on a rocking platform. Membranes were washed two times in TBS-T and subsequently incubated with goat anti-rabbit, peroxidase-conjugated secondary antibody (Sigma; 1:2000 in Blotto B) for 30 min at room temperature on a rocking platform. The membranes were then washed 3X in TBS-T and once in TBS, and immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL, U.S.A.) using Kodak X-OMAT film (Sigma). Immune complexes were quantified by densitometry using a Bio-Rad Model GS-670 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

In some experiments, membranes were stripped and reprobed with a different anti-PKC antibody. Stripping was done by immersing membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)) for 30 min at 50°C in a shaking water bath. After stripping, the membranes were washed 2X for 10 min in TBS-T. Following these washes, membranes were handled as described above, starting at the point of blocking. Membranes were not stripped more than twice.

In addition to the C-terminal anti-PKC- δ antibody described above, a second PKC- δ antibody was employed to evaluate the expression of this isozyme in the normal osteoblasts and the osteoblastic cell lines. This second antibody was a mouse monoclonal antibody raised against a peptide derived from the internal portion of the PKC- δ molecule (amino acids 114-289, rat PKC- δ) (Transduction Laboratories, Lexington, KY, U.S.A.). Western blots with this antibody were carried out as described above, except that membranes were blocked with 5% Carnation non-fat dry milk (in PBS-T). Both the primary and the secondary antibody (goat anti-mouse; Sigma) were made up in this blocking buffer (1:1000).

Results

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Task 1: Determination of the presence of PKC- γ , ϵ , and η /L isozymes in UMR-106 cells.

The studies set forth in Task 1 were accomplished, and the results are described below. In the course of these studies, however, it became clear that a broader determination of PKC isozyme expression in osteoblasts was necessary, not only in terms of the proposed studies but to serve as a foundation for future studies involving PKC isozymes and their role(s) in regulating bone remodeling. In addition, numerous studies were carried out to characterize the sensitivity of the PKC isozymes in osteoblasts to phorbol ester-induced down-regulation. This characterization is important for at least two reasons: 1. Knowledge of the phorbol sensitivity of the PKC isozymes in osteoblasts provides a means of comparing/contrasting the osteoblastic isozymes to the same isozymes expressed in other cell types and/or tissues. 2. We plan to use phorbol esters in future studies to examine the involvement of PKC in hormone-mediated changes in osteoblast function. To obtain interpretable information from such studies, however, we must have knowledge of the precise effects of the phorbol ester on the various PKC isozymes.

PKC isozyme expression in normal osteoblasts and osteoblastic cell lines

To determine if there is a characteristic pattern of PKC isozyme expression in osteoblasts, the isozyme profiles of eight different osteoblasts/osteoblast-like cell lines were elucidated by Western immunoblotting. The osteoblasts screened included normal neonatal mouse osteoblasts, three rat osteoblastic cell lines (UMR-106, ROS 17/2.8, ROS 24/1), and four human osteoblastic cell lines (MG-63, G-292, SaOS-2, HOS-TE85). Figure 2 shows the Western blots obtained utilizing antibodies to the conventional PKC isozymes, α , β_I , β_{II} , and γ ; PKC- β_I and β_{II} are derived from a single gene by alternative splicing and differ only in their carboxy-termini (approximately 50 amino acids)⁽³⁶⁾. PKC- α and β_I were expressed in each of the osteoblasts examined; although the reactivity was low, PKC- β_{II} was detectable in all but the ROS 24/1 rat line. The γ isozyme, however, was not detectable in any of the osteoblasts screened.

The molecular weights of the conventional PKC isozymes detected in osteoblasts were consistent with those reported for these isozymes in other tissues⁽³⁸⁾. PKC- α , β_I and β_{II} each migrated with an apparent molecular weight of approximately 80-82 kilodaltons (kDa). β_I was detected as a doublet in nearly all of the osteoblasts examined (Figure 2). This doublet may be represent different phosphorylation forms of the β_I isozyme, as reported for several of the PKC isozymes⁽³⁸⁾.

The novel isozymes examined in osteoblasts were PKC- δ , ϵ , η , and θ (Figure 3). Like the conventional α and β_I isozymes, PKC- ϵ was expressed in each of the osteoblasts screened; the immunoreactivity of ϵ differed widely, however, among the different osteoblasts. The other novel isozymes showed more varied expression between cell lines. Utilizing the anti-PKC- δ antibody raised against a peptide derived from the internal portion of PKC- δ , this isozyme was readily observable in the UMR-106 rat line, and minimally detectable in normal mouse osteoblasts and in the SaOS-2 and HOS-TE85 human lines (Figure 3). Using the C-terminal PKC- δ antibody, δ was

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also readily detectable in the UMR-106 cells; among the other osteoblasts, δ was evident only in the normal osteoblasts (data not shown). PKC- η was expressed in the normal mouse osteoblasts, the UMR-106, SaOS-2 and HOS-TE85 cells and faintly in the ROS 24/1 and MG-63 lines; it was not found in the ROS 17/2.8 cells. PKC- θ was expressed only in the SaOS-2 and HOS-TE85 lines; on some blots, θ was also detectable in the ROS 24/1 line (data not shown).

As was found for the conventional isozymes, the molecular weights of the novel PKC isozymes detected in osteoblasts were consistent with those reported for these isozymes in other tissues^(38,39-41,48). PKC- δ migrated with a molecular weight of approximately 76 kDa. PKC- ϵ ran at approximately 90 kDa and like β_1 , was detected as a doublet in some of the osteoblasts examined (Figure 3). PKC- η appeared as a doublet in the human cell lines in which it was expressed. The lower band of these doublets migrated to the same extent as did the single band observed in the other η -expressing osteoblasts (Figure 3). The lower band corresponded to 75 kDa and the upper band to 81 kDa. PKC- θ had a molecular weight of approximately 79 kDa.

Expression of the atypical ζ and ι/λ isozymes was also examined in the osteoblasts. Because PKC- ζ and ι/λ share a common C-terminus^(42,43), the C-terminal PKC- ζ antibody cross-reacts with PKC- ι/λ , and both isozymes are seen on the same blot. PKC- ζ was detected as a single band at approximately 75 kDa and PKC- ι/λ as a single band at approximately 65 kDa; these molecular weights agree with those reported for these isozymes in other tissues⁽⁴²⁾. As shown in Figure 4, both isozymes were expressed in each of the osteoblasts screened. The highest molecular weight band (C, 80 kDa) seen on this blot may represent a conventional isozyme, possibly PKC- α ; cross-reactivity of C-terminal ζ antibodies with conventional isozymes has been reported previously^(49,50). As was observed with the PKC- α -specific antibody (Figure 2), this isozyme was expressed in each of the osteoblasts screened.

Sensitivity of PKC isozymes to prolonged phorbol ester treatment

Phorbol esters are widely employed as pharmacological tools to explore the role of PKC in cellular processes. To obtain interpretable information from the physiological results, however, one must have knowledge of which isozymes the phorbol ester acts on as well as the time course over which these effects are manifested. To characterize the action of phorbol esters in osteoblasts, the effect of 1-48 h phorbol ester treatment of the cells on PKC isozyme expression was examined. Normal mouse osteoblasts and the UMR-106 rat osteoblastic cells were selected for these time course studies. As shown in Figure 5, the time courses and the extent of the responses of the conventional α and β_1 isozymes to 1 μ M PDB differed somewhat in the normal mouse osteoblasts and the UMR-106 osteoblastic cells; the sensitivity of the β_{II} isozyme was not examined due to the relatively low expression of this isozyme in osteoblasts. In the normal osteoblasts, slight down-regulation of PKC- α was detectable after only a 1 h treatment with 1 μ M PDB (77% of 1 h control. Note: Time course calculations are based on time-matched controls). Down-regulation of PKC- β_1 was first evident at 3 h, having decreased to 64% of control. At 6 h, both PKC- α and β_1 fell to approximately 48% of control levels. Down-regulation of both isozymes was maintained at approximately 50% at all later time points examined (Figure 5B).

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In the UMR-106 line, down-regulation of PKC- α and - β_1 was first detectable following 3 h treatment with 1 μ M PDB (Figure 5); at 3 h, α and β_1 were decreased to $78 \pm 4\%$ (mean \pm standard error) and $71 \pm 7\%$ of control, respectively (Figure 5B). At 6 h, both isozymes were down-regulated to a slightly greater extent, falling to $63 \pm 4\%$ (α) and $63 \pm 13\%$ (β_1) of control levels. PKC- α continued to decline to $31 \pm 6\%$ and $18 \pm 4\%$ of control at 12 and 24 h, respectively, but then reached a plateau ($17 \pm 8\%$ of control) with 48 h PDB treatment. Levels of PKC- β_1 also continued to decline with longer treatment periods, dropping to $38 \pm 15\%$, $23 \pm 7\%$, and $16 \pm 4\%$ of control at 12, 24, and 48 h, respectively.

The novel isozymes showed a range of sensitivities to 1 μ M PDB treatment, with the δ and ϵ isozymes being more completely down-regulated than PKC- η (Figure 6). In the normal osteoblasts, PKC- δ was dramatically down-regulated after 1 h PDB treatment (56% of control) and further down-regulated at 3 h (24% of control). In examining the Western blot, PKC- δ appears to be completely down-regulated at all subsequent time points. The densitometer, however, detects some residual immunoreactivity at these later time points. PKC- ϵ was also completely down-regulated by prolonged phorbol treatment in normal osteoblasts, although the time course was somewhat different than for δ . Down-regulation of PKC- ϵ was first apparent at 6 h and was complete at this time point. As for PKC- δ , down-regulation of PKC- ϵ was maintained at all subsequent time points.

Expression of the δ and ϵ isozymes in UMR-106 osteoblastic cells was affected in a parallel manner by prolonged PDB treatment (1 μ M) (Figure 6). PKC- δ was partially down-regulated after 1 h PDB treatment ($68 \pm 3\%$ of control), further down-regulated at 3 and 6 h ($18 \pm 5\%$ and $11 \pm 3\%$ of control, respectively), and completely down-regulated at all subsequent time points ($<5\%$ of control). Down-regulation of PKC- ϵ was first detectable in the UMR-106 cells at 3 h ($62 \pm 6\%$ of control), and declined progressively with 6, 12, 24, and 48 h treatments (30 ± 6 , $15 \pm 4\%$, $12 \pm 2\%$, and $7 \pm 1\%$ of control, respectively).

The novel η isozyme was unique in that it was affected by phorbol ester treatment in two distinct ways. These effects were seen in both the normal mouse osteoblasts and the UMR-106 cell line. First, phorbol treatment caused a slight decrease in the mobility of PKC- η (Figure 6A). This effect, seen as a slight upward shift in the PKC- η band, was apparent at all time points examined and may be due to phosphorylation of η with phorbol treatment. Second, phorbol treatment caused a down-regulation of the η isozyme (Figure 6A, B), as described above for the other novel isozymes. In normal osteoblasts, PKC- η was only moderately sensitive to prolonged PDB treatment. Down-regulation was apparent at 6 h (75% of control) but failed to progress with longer treatment periods. In contrast, in the UMR-106 line, down-regulation of η was first detectable at 6 h ($84 \pm 27\%$ of control) and declined further at later time points, falling to $43 \pm 12\%$, $38 \pm 12\%$, and $28 \pm 3\%$ of control levels at 12, 24, and 48 h, respectively.

Unlike the conventional and novel PKC isozymes, the atypical ζ and ι/λ isozymes were insensitive to prolonged phorbol ester treatment at all time points examined (Figure 7A, B). This insensitivity was observed in both the normal mouse osteoblasts and the UMR-106 cell line. As seen in Figure 4, the uppermost band observed in blots with the anti-PKC ζ antibody (C) was down-regulated by phorbol ester treatment. In the normal osteoblasts, down-regulation was detectable after 1 h PDB treatment (69% of control). Isozyme levels were slightly higher at 3 h but

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were still lower than in control cells (81% of control). At 6 h, levels fell to 38% of control, and remained at 45-60% of control at all subsequent time points. In the UMR-106 cells, down-regulation of this isozyme was apparent after 3 h PDB treatment ($78 \pm 5\%$ of control). Isozyme levels declined progressively at later time points falling to $65 \pm 10\%$, $40 \pm 6\%$ and $26 \pm 7\%$ of control at 6, 12, and 24 h, respectively. The down-regulation observed at 24 h was maintained with 48 h PDB treatment ($24 \pm 6\%$ of control). These results are consistent with those obtained using the PKC- α -specific antibody (Figure 5).

The time course studies described above were carried out with a single dose (1 μM) of the phorbol ester PDB. This suggested that the more moderate down-regulation observed for the conventional as compared to the novel isozymes could be a dose-related effect. Moreover, it was possible that the lack of down-regulation of the atypical ζ and ι/λ isozymes was dose related. To address this issue, the sensitivity of the isozymes to 24 or 48 h treatment with two additional doses of PDB was examined. The conventional (α , β_1) and novel (ϵ , η) isozymes showed a greater degree of down-regulation following 48 h treatment with 1 μM versus 0.3 μM PDB; PKC- δ and $-\eta$ were maximally down-regulated even with 0.3 μM PDB (Figure 8). Following treatment with 3 μM PDB, the conventional and novel isozymes were down-regulated to approximately the same extent as with 1 μM PDB treatment. As was observed with 1 μM PDB, 48 h treatment with 0.3 μM or 3 μM PDB failed to down-regulate the atypical PKC isozymes. Similar dose-response results were obtained following 24 h treatment with 0.3 μM , 1 μM , or 3 μM PDB (data not shown).

To examine the specificity of the phorbol ester-mediated down-regulation, experiments were carried out with the inactive phorbol ester 4 α -phorbol 12,13-didecanoate (α -PDD). 48 h treatment of UMR-106 cells with 1 μM α -PDD had no effect on expression of the conventional, novel, or atypical isozymes as compared to vehicle-treated cells (data not shown).

Phorbol ester-mediated down-regulation of PKC is associated with an increased rate of degradation of PKC⁽⁵¹⁾. This down-regulation has been reported to be reversible in other cell types⁽⁵²⁾. To examine the reversibility of the PDB-mediated down-regulation in osteoblasts, UMR-106 cells were exposed to vehicle for 48 h, 1 μM PDB for 48 h, or 1 μM PDB for the first 24 h and vehicle for the final 24 h. The phorbol-sensitive conventional (α , β_1) and novel (δ , ϵ , η) isozymes were each significantly down-regulated by 48 h PDB treatment (Figure 9), as was seen in the time course and dose response studies. When the PDB was removed at 24 h, however, all isozymes, except PKC- η , had recovered some extent by 48 h. PKC- α was restored nearly to control levels, while PKC- β_1 , $-\delta$, and $-\epsilon$ recovered to 40-70% of control. The phorbol-sensitive isozyme detected with the anti- ζ antibody (C) recovered to 63% of control.

Task 2: Characterization of the effects of 17 β -estradiol and tamoxifen on PKC isozyme expression in UMR-106 cells.

By determining which of the PKC isozymes are commonly expressed in osteoblasts, we obtained important information to guide the studies outlined in this task. As mentioned above, PKC- α , β_1 , ϵ , ζ , and ι are expressed in all of the osteoblasts examined. Based on this information, each of our estrogen and tamoxifen studies included an assessment of the expression of these

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isozymes. The expression of PKC- η and - θ was not examined in the estrogen/tamoxifen studies because they are expressed in only some of the osteoblasts examined; furthermore, these isozyme are relatively difficult to detect. Like η and θ , PKC- δ was expressed in only some of the osteoblasts examined. We did examine the expression of this isozyme in the estrogen/tamoxifen studies, however, because δ has been shown to be estrogen responsive in other tissues^(23,24).

Since the effects of 17 β -estradiol and tamoxifen on PKC isozyme expression have not been previously studied in bone, we first explored a range of hormone treatment protocols. Described below are the numerous treatment strategies that have been employed to determine if 17 β -estradiol or tamoxifen affect PKC isozyme expression in UMR-106 cells. Unless otherwise noted, all treatments were carried out in phenol red-free medium with charcoal-stripped serum to reduce the effects of endogenous estrogens.

The hormone treatment strategy initially proposed involved a 24 h treatment of confluent UMR-106 cells with vehicle or hormone (17 β -estradiol, 4-OH-tamoxifen; 10^{-12} to 10^{-7} M). These treatments followed a 24 h period in which the cells were maintained in phenol red-free medium with charcoal-stripped serum. The Western blots and the corresponding densitometric analysis of the blots for PKC- α and ϵ are shown in Figure 10. Although small changes in both of these isozymes were seen, neither exhibited greater than a two-fold change in expression, which was the degree of change that was taken to be significant throughout these studies. Despite repeated efforts, blots for PKC- β_1 , - ζ , and - ι showed only faint bands; as a result, densitometric analysis of the blots for these isozymes was not possible.

The next treatment strategy that was utilized extended the time course of the treatment strategy described above. In addition to the 24 h treatment period initially employed, confluent UMR-106 cells were treated for 48 and 72 h with 17 β -estradiol (10^{-10} to 10^{-8} M). In addition, the pre-treatment period in phenol red-free medium (with charcoal-stripped serum) was extended to 72 h to enhance the likelihood of detecting a response. The Western blots and the densitometric analysis of the blots for PKC- α , β_1 , δ , ϵ , ζ , and ι are shown in Figure 11. As for the initial treatment strategy, no significant effect of 17 β -estradiol on PKC isozyme expression was observed. PKC- β_1 showed an apparent increase in expression at 24 h at the two highest concentrations of 17 β -estradiol, but then at 48 h, β_1 was reduced to approximately 50% of control. These changes could conceivably be artifacts of the blot; PKC- ϵ , visualized on the same blot, showed a similar pattern of expression (higher than control at 24 h, lower at 48 h, approximately the same as control levels at 72 h). Furthermore, the apparent effects on PKC- β_1 were not dose dependent. A similar situation was observed for PKC- ζ at 24 h; the densitometric analysis indicated that ζ increased to nearly twice control levels following 24 h treatment with 17 β -estradiol. In looking at the blot, however, the 24 h control band for ζ appears to be incomplete, particularly the left side of the band. These studies need to be repeated to examine the reproducibility of the findings. Tamoxifen was not included in this study because of the greater number of time points employed.

The two studies described above both involved treating confluent UMR-106 cells with estrogen and/or tamoxifen. Because the degree of confluence has been shown to affect the response of UMR-106 cells to estrogen⁽⁵³⁾, subsequent experiments were carried out with subconfluent cells. In one such experiment, UMR-106 cells were treated with 1 nM 17 β -estradiol

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for 1, 1.5, 2, 3, 4, or 5 days. The cells remained subconfluent throughout the course of the experiment and at the time of harvest. The Western blots for the samples obtained in this experiment are shown in Figure 12 as are the corresponding graphs for the densitometric analysis. Though some fluctuations in isozyme expression were seen at various time points in this study, there did not appear to be any time-dependent effects on expression. Furthermore, the observed changes all differed less than two-fold from the appropriate time-matched controls.

Because of the unconvincing results obtained in the first three experiments, we sought a different approach to estrogen treatment for the next set of experiments. Passeri *et al.*⁽⁵⁴⁾ recently demonstrated that 17β -estradiol inhibits interleukin-1(IL-1)- and tumor necrosis factor(TNF)-induced interleukin-6 (IL-6) production in numerous osteoblast models. In addition, this group showed that addition of 17β -estradiol to calvarial cell cultures followed by withdrawal of the steroid increased the amount of IL-6 produced in response to subsequent stimulation of the cultures with IL-1 or parathyroid hormone (PTH) (compared to that produced in cultures that had never been treated with 17β -estradiol). No change in IL-6 production was observed when 17α -estradiol was used.

Based on the results of this study, we chose to examine the effects of a similar estrogen withdrawal paradigm on PKC isozyme expression in UMR-106 osteoblastic cells. UMR-106 cells were seeded in DME containing phenol red and 15% heat-inactivated horse serum. After 24 h, the medium was changed to phenol red free DME with 15% charcoal-stripped, heat-inactivated horse serum containing either vehicle or 17β -estradiol (10^{-10} to 10^{-8} M). After 3 days, the medium was removed, the cells were washed two times with phenol red-free DME, and fresh medium containing treatments was added to the cells for an additional 3 days. Of the four dishes initially treated with vehicle, one was continued with this treatment while the other 3 were treated with 17β -estradiol (10^{-10} , 10^{-9} , or 10^{-8} M). One of the two dishes that had been treated with 10^{-10} M 17β -estradiol for the first 3 days continued to receive this treatment while the other dish was switched to vehicle treatment; the same pattern was followed for those cells initially treated with 10^{-9} and 10^{-8} M 17β -estradiol. The treatments described were necessary to provide the appropriate controls. As a result, the estrogen treatments included not only the 3 day estrogen withdrawal following 3 day estrogen treatment, but also estrogen treatment for only the last 3 days of the experiment and continual estrogen treatment for 6 days.

The results of the estrogen withdrawal experiment are shown in Figure 13; the Western blots for each of the isozymes are shown in Figure 13A, and the data from the densitometric analysis of the blots is presented in tabular form in Figure 13B. PKC- α , β_1 , δ , and ζ were not affected by any of the 17β -estradiol treatments employed in this study. The expression of PKC- ϵ and PKC- ι , however, did appear to be altered. PKC- ϵ was increased in a dose-dependent manner following continual, 6 day estrogen treatment, rising to 111%, 117%, and 132% of control with 10^{-8} , 10^{-9} , and 10^{-10} M 17β -estradiol, respectively. PKC- ϵ was increased in a similar fashion by estrogen withdrawal; ϵ was increased to 113, 125%, and 138% of the vehicle control 3 days after withdrawing 10^{-8} , 10^{-9} , and 10^{-10} M 17β -estradiol, respectively.

Like PKC- ϵ , the expression of PKC- ι was increased following both 6 day estrogen treatment and estrogen withdrawal. There were at least two important differences between the effects observed for ϵ and ι , however. First, the dose-dependence of the effect of 6 day estrogen

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treatment on ι was not as clear as that observed for ϵ . Second, while estrogen withdrawal did dose-dependently increase the expression of PKC- ι , the dose effect was opposite to that observed for ϵ ; in other words, the expression of ι was increased to the greatest extent at the highest estrogen concentration used, whereas the greatest effect on ϵ was observed at the lowest estrogen concentration used.

The effects of estrogen treatment on PKC- ϵ and ι described above were not significant according to our aforementioned criterion requiring at least a two-fold change in expression. The estrogen withdrawal experiments provided the first clear indication of a dose-dependent effect, however, suggesting that we had found an effective treatment strategy but had not used maximally effective concentrations of 17 β -estradiol. To determine if this was in fact the case, we repeated the estrogen withdrawal experiment but used lower concentrations of 17 β -estradiol in addition to some of the concentrations used in the initial estrogen withdrawal experiment (10^{-12} to 10^{-9} M). We were most interested in any effects on PKC- ϵ , given the dose-dependence observed in the previous experiment. No significant effects on PKC- ϵ were observed in the second experiment, however, even at the lowest concentrations of 17 β -estradiol (Figure 14). The expression of PKC- β_1 , ζ , and ι was also examined in this second estrogen withdrawal experiment; as for PKC- ϵ , no significant changes were observed.

The next treatment strategy that was employed was one that has been shown to decrease the expression of PKC- δ in MCF-7 human breast cancer cells (M. Shanmugan, personal communication). Cells were seeded in media containing both phenol red and serum and allowed to attach overnight. The next day, the cells were washed, and the medium was changed to phenol red-free medium with charcoal-stripped serum. After 4 days in this estrogen-depleted medium, fresh medium containing either vehicle, 17 β -estradiol (10^{-12} to 10^{-9} M), or 4-OH-tamoxifen (10^{-8} to 10^{-6} M) was added to the cells. The treatments were continued for 7 days, with medium replacements every 3 days. The Western blots for the samples obtained in this experiment are shown in Figure 15 as are the corresponding graphs for the densitometric analysis. PKC- α , δ , ϵ , ζ , and ι were unaffected by treatment with either 17 β -estradiol or tamoxifen. PKC- β_1 was not affected by 17 β -estradiol, but exhibited a dose-dependent increase in expression following treatment with tamoxifen. At the two highest doses of tamoxifen, the increase in PKC- β_1 was greater than two-fold over control. Because these last experiments were completed quite recently, there was not time to repeat them prior to the submission of this report.

The final hormone treatment strategy that was utilized was one that has been shown to affect other osteoblastic parameters, including cell proliferation and enzyme activity⁽⁵⁵⁾. UMR-106 cells were seeded in DME:F12 (1:1) containing 5% FBS. After 24 h, the medium was changed to phenol red-free DME:F12 (1:1) containing insulin, transferrin, and selenium (ITS). 24 h later, fresh medium containing either vehicle, 17 β -estradiol (10^{-11} to 10^{-9} M), or 4-OH-tamoxifen (10^{-9} to 10^{-7} M) was added to the cells. The cells were harvested 72 h later. The Western blots and the corresponding densitometric analysis for PKC- β_1 and ϵ are shown in Figure 16; because this experiment was performed quite recently, these are the only isozymes for which blots were obtained. The expression of PKC- β_1 in 17 β -estradiol-treated cells was approximately 2-fold higher than that observed in control cells, but this level of expression was observed at all doses of estrogen tested. As in the previous experiment, the expression of PKC- β_1

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was increased substantially by tamoxifen treatment. The increase observed in this experiment was greater than that in the previous experiment, however, with a maximal increase of nearly 5-fold over control. The expression of PKC- ϵ was not significantly affected by treatment with either 17 β -estradiol or tamoxifen. These studies will be repeated to examine the reproducibility of the findings.

Task 2 also included a determination of the effects of 17 β -estradiol and tamoxifen on mRNA levels for any isozymes affected by hormone treatment. As no definitive changes in isozyme levels were discernible at the protein level, however, these studies were not performed.

Task 3: Determination of the effects of other sex steroids (17 α -estradiol, progesterone, testosterone) on PKC isozyme expression.

The studies set forth in Task 3 were to be performed assuming that an effect of 17 β -estradiol and/or tamoxifen on PKC isozyme expression was found. If such an effect were evident, the studies outlined in this task would then provide a means of characterizing the hormonal specificity of the 17 β -estradiol/tamoxifen effect. As no definitive effect of 17 β -estradiol or tamoxifen on isozyme expression was found, studies with 17 α -estradiol, progesterone, and testosterone were not performed.

Task 4: Characterization of the effects of tamoxifen/sex steroids on PKC isozyme expression in the human osteoblastic cell lines SaOS-2 and MG-63 and in primary mouse osteoblasts.

The studies included in Task 4 were to be carried out assuming that an effect of 17 β -estradiol and/or tamoxifen on PKC isozyme expression was found in the UMR-106 rat osteoblastic cell line. If such an effect were clear in the UMR-106 cells, similar studies with human osteoblastic cell lines and primary mouse osteoblasts would provide a means of addressing the physiological significance of the results obtained with the UMR-106 cells.

Studies with human osteoblastic cells and primary osteoblasts will be carried out, but the purpose of these studies may change. The purpose will change if continued efforts to find an effect of estrogen and/or tamoxifen on PKC isozyme expression in UMR-106 cells fail. If no effect is found, the goal of studies with the human cells and the primary osteoblasts will be to determine if a hormonal effect on PKC isozyme expression can be elicited in osteoblasts. Such an effect may be evident in one or both of the human cell lines and/or in the primary osteoblasts, but not in the UMR-106 cells, due to differences in the state of differentiation of these osteoblasts⁽⁵⁶⁻⁶⁰⁾.

Studies with the human osteoblastic cell lines have been initiated; MG-63 and SaOS-2 cells were cultured and the former selected for initial studies. MG-63 cells were seeded and treated according to the originally proposed treatment strategy (24 h treatment of cells with vehicle, 17 β -estradiol, or tamoxifen). These cells have been harvested and prepared for electrophoresis.

Discussion

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Expression and Phorbol Ester-induced Down-regulation of PKC Isozymes in Osteoblasts

The current results indicate that there is a characteristic pattern of PKC isozyme expression in osteoblasts across species and in both normal and osteosarcoma-derived cells. The PKC isozymes found to be present in all of the osteoblasts screened are PKC- α , - β_I , - ϵ , - ζ and - ι/λ (Figures 2-4). PKC- β_{II} is expressed in each of the osteoblasts except the ROS 24/1 rat line, which is less osteoblastic than the other cell lines examined^(45,61). The novel δ , η , and θ isozymes are detectable only in some of the osteoblasts examined. The variability in expression of these isozymes may be a reflection of the different stages of differentiation of the osteoblasts screened⁽⁵⁶⁻⁶¹⁾. Conversely, the variable expression of PKC- δ , - ϵ and - η may be an indication that these isozymes are not important for the uniquely osteoblastic functions of these cells.

Few studies of PKC isozyme expression in bone have been conducted. Sakai *et al.*⁽⁶²⁾ examined conventional PKC isozyme expression in MC3T3-E1 mouse osteoblastic cells. MC3T3-E1 cells, like the osteoblastic cells examined in our study, express PKC- α and - β , but not γ (the β antibody used detected both β_I and β_{II}). Isozyme expression in osteoclast-like cells has also been partially characterized. Teti *et al.*⁽⁶³⁾ examined isozyme expression in both human osteoclast-like cells (isolated from giant cell tumors of bone) and freshly isolated rat osteoclasts. Both types of osteoclast-like cells express PKC- α , - δ , and - ϵ , but not β . (As in the Sakai *et al.* study, the anti-PKC- β antibody was capable of detecting both β_I and β_{II}). Expression of the other known PKC isozymes was not determined.

The study presented here is the first thorough characterization of the isozymes present in osteoblastic cells and indicates the difference between bone and other tissues, such as brain, which expresses all the known isozymes of PKC⁽³⁶⁻⁴³⁾ and kidney which expresses PKC- α , - δ , - ϵ , and - ζ ⁽³⁸⁾. The α , β_I/β_{II} , δ , ϵ , and ζ isozymes are ubiquitously expressed^(37,38), and of these, all but PKC- δ are commonly expressed in osteoblasts. None of the osteoblasts screened express PKC- γ . This result is not surprising, however, as this isozyme has been detected primarily in the central nervous system^(36,37). A broad determination of the tissue expression of the other isozymes examined in this study, PKC- η , - θ , and - ι/λ has not yet been carried out. Initial studies suggest that PKC- η and - θ have a rather limited distribution, with η being expressed predominantly in the skin and lung^(39,40) and θ largely in skeletal muscle⁽⁴¹⁾. Both isozymes are expressed at a lower extent in the brain and spleen⁽³⁹⁻⁴¹⁾. In contrast to η and θ , initial studies of PKC ι/λ expression suggest that this isozyme is widely expressed, but most notably in the lung, brain, and kidney^(42,43).

As expected, there are differences in phorbol ester sensitivity among the different osteoblast PKC isozymes (Figures 5-7). The conventional and novel isozymes are down-regulated by prolonged phorbol ester treatment, but the atypical isozymes are not. These results are consistent with the ability of these classes to bind phorbol esters. The conventional and novel isozymes possess the two cysteine-rich zinc finger motifs in the conserved C1 PKC domain necessary to bind DAG and phorbol esters⁽³⁶⁻³⁸⁾; atypical isozymes lack one of the two zinc fingers, however, and are unable to bind DAG or phorbol esters^(42,43,64-67).

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Down-regulation occurs quite rapidly for nearly all of the phorbol-sensitive osteoblast isozymes (Figures 5-7). All sensitive isozymes, with the exception of PKC- η in normal osteoblasts, are significantly diminished following 6 h phorbol treatment. This rapid time course of down-regulation has implications for experiments in which a phorbol ester is used as a tool to presumably activate PKC. Unless the exposure times are very short, the results may actually be due to a down-regulation rather than an activation, leading to an opposite interpretation for the role of PKC in the process. Another possibility in such experiments is that the initial activation, rather than the subsequent down-regulation of the kinase activity, is the critical event.

Although both the conventional and novel isozymes are down-regulated by prolonged phorbol treatment in osteoblasts, the extent of the down-regulation observed for these two classes is somewhat different (Figures 5,6). The conventional α and β_I isozymes are less sensitive to prolonged phorbol ester treatment than the novel isozymes, particularly PKC- δ and $-\epsilon$. This difference in sensitivity does not appear to be a dose-related effect as 24 or 48 h treatment with 3 μ M PDB produces approximately the same degree of down-regulation as 1 μ M PDB (Figure 8). Treatment with a lower dose of PDB, however, is less effective in down-regulating some isozymes (Figure 8), indicating that phorbol-induced down-regulation is a concentration-dependent effect in osteoblasts. Another possibility is that the conventional isozymes might be further down-regulated with longer phorbol treatments (>48 h); this seems unlikely, though, because PKC- α and β_I isozyme levels appear to plateau with 24 and 48 h phorbol treatments (Figure 5). Differences in the extent of phorbol-induced down-regulation of conventional and novel isozymes have been reported previously⁽⁶⁸⁾, but the reason for this difference is not clear.

The phorbol ester-induced down-regulation of PKC isozymes observed in osteoblasts is a specific and reversible effect. Evidence of the specificity is provided by studies with the inactive phorbol α -PDD, which fails to down-regulate PDB-sensitive isozymes (data not shown). In addition, the atypical isozymes are insensitive to the phorbol treatments that significantly down-regulate the conventional and novel isozymes (Figure 7). The reversibility of the phorbol-induced down-regulation observed in osteoblasts is consistent with the means by which this down-regulation is effected. Prolonged phorbol treatment increases the net rate of degradation of PKC⁽⁵¹⁾. When phorbol is removed from the cellular environment, however, degradation declines to its basal rate and due to the ongoing synthesis of PKC, isozyme levels are eventually restored. The precise molecular mechanism(s) responsible for the phorbol-induced down-regulation of PKC is not known.

As mentioned above, the failure of prolonged phorbol treatment to down-regulate the atypical ζ and ν/λ isozymes in osteoblasts is consistent with the inability of these isozymes to bind phorbol ester^(43,64-67). A similar insensitivity of PKC- ζ to phorbol has been described in other cell types, including renal mesangial cells⁽⁵²⁾, melanocytes^(68,69), and Jurkat T lymphoma cells⁽⁷⁰⁾. Phorbol-induced down-regulation of ζ has also been reported⁽⁷¹⁻⁷³⁾, but in many of these studies, the authors employed a C-terminal PKC- ζ antibody, as used in our studies. For example, in a recent study carried out in urinary tract smooth muscle cells⁽⁷³⁾, the cross-reactive anti-PKC- ζ antibody was utilized to examine the sensitivity of the ζ isozyme to 24 h phorbol myristate acetate (PMA) treatment. As in our studies, the Western blot revealed three bands at approximately 80, 75, and 65 kDa. The 80 kDa ζ -reactive band was down-regulated by 24 h PMA treatment, but the

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other two bands were unaffected by this treatment. The 80 kDa band was considered to be PKC- ζ , and no mention was made of the other two bands observed on the blot. Therefore, when results obtained with C-terminal PKC- ζ antibodies are interpreted, the ability of this antibody to cross-react with phorbol-sensitive conventional isozymes must be considered.

Effect of 17 β -Estradiol and Tamoxifen on PKC Isozyme Expression in UMR-106 Cells

Although no definitive effect of estrogen or tamoxifen on PKC isozyme expression has been found, the results obtained in the two most recent experiments in UMR-106 cells look promising. In both studies, tamoxifen increased the expression of PKC- β_1 ; the changes in expression that were observed in these experiments were quite a bit greater than those observed in any of our previous estrogen and/or tamoxifen studies. Additional experiments will be necessary to verify the effects of tamoxifen under these treatment conditions. If the effects are legitimate, the responses will then be further characterized, which will include, in part, a comprehensive determination of their time- and dose-dependence.

While we remain hopeful concerning the experiments described above, the large amount of unconvincing data that we have generated has forced us to consider the possibility that estrogen and tamoxifen do not alter PKC isozyme expression in osteoblasts. A negative result is never definitive, however, because there is always the possibility that the "right" treatment conditions have not been found. One possibility is that these hormones do alter isozyme expression, but they do so only over more extended treatment periods than were utilized here. Such a requirement for extended treatment periods is consistent with the relatively extended period of time over which estrogen deficiency-induced bone loss occurs. Postmenopausal bone loss occurs gradually over several years, and changes at the molecular level in bone may occur gradually as well. If this is the case, it would be very difficult to detect changes in isozyme expression after a period of only a week or less of hormone treatment.

A second possible explanation for the difficulty in detecting any hormone-induced changes in isozyme expression may relate to the pattern of isozyme expression that was found in osteoblasts. The expression of PKC- δ , but not other PKC isozymes, has been shown to be regulated by estrogen in both the rabbit⁽²³⁾ and the rat⁽²⁴⁾ ovary and in MCF-7 human breast cancer cells (M. Shanmugan, personal communication). This suggests that δ may be the primary estrogen-sensitive PKC isozyme. PKC- δ was not detected in all of the osteoblasts that were screened, however. Because PKC- δ is not commonly expressed in osteoblasts, it may be less likely that an estrogen effect on isozyme expression exists in this cell type.

A third possibility for the difficulty that we have encountered in trying to find an effect of estrogen and/or tamoxifen on PKC isozyme expression may relate to the state of differentiation of the osteoblasts being studied; in other words, the hormonal effect on isozyme expression may only occur at a certain stage or stages of osteoblastic differentiation. This issue will be addressed in the studies described in Task 4. In these studies, we will determine whether estrogen and/or tamoxifen regulate PKC isozyme expression in two human osteoblastic cell lines and in primary (normal) mouse osteoblasts, which represent different stages of osteoblastic differentiation⁽⁵⁶⁻⁶⁰⁾.

There is another explanation for our difficulties that must be considered; that is, estrogen and/or tamoxifen may alter PKC isozyme expression in osteoclasts, not osteoblasts. We plan to address this possibility, but will have to use a different approach than was used in the osteoblast experiments. An alternative approach is necessary because there are few osteoclastic cell lines; in addition, normal osteoclasts are difficult to isolate from bone because there are few osteoclasts relative to osteoblasts. One way to examine the effect of estrogen and tamoxifen on isozyme expression in osteoclasts involves the use of intact mouse calvaria, which contain both osteoblasts and osteoclasts. Calvaria can be maintained in 24 well plates (1 bone/well) in cell culture medium, and treatments (vehicle, 17β -estradiol, tamoxifen) added to each well. After the treatment period, the bones are homogenized and samples are prepared for electrophoresis and subsequent Western blotting. Utilizing this approach, we have been able to characterize the isozymes in calvarial homogenates; all of the isozymes that are common to osteoblasts, PKC- α , β_1 , ϵ , ζ , and ι , were detected. If these calvarial experiments reveal an effect of estrogen and/or tamoxifen on isozyme expression, additional experiments, such as those described below, would be necessary to verify that this was an effect on the osteoclast.

Another, more direct means of exploring whether estrogen and/or tamoxifen regulate PKC isozyme expression in osteoclasts involves the use of bone marrow cell cultures, which contain osteoclast precursors. When these marrow cells are cultured in the presence of 10^{-7} to 10^{-8} M 1,25-dihydroxy-vitamin D_3 , the osteoclast precursors differentiate into mature osteoclasts. These osteoclasts can then be treated with estrogen/tamoxifen and isozyme expression can be examined.

CONCLUSIONS

These studies, which are the first to thoroughly characterize PKC isozyme expression in osteoblastic cells, demonstrate that osteoblasts have a characteristic PKC isozyme profile, including both phorbol ester-sensitive and -insensitive isozymes. The phorbol-sensitive isozymes are those of the conventional and novel classes, while the insensitive isozymes correspond to the atypical class. Down-regulation of all phorbol-sensitive isozymes was detectable within 6 h of phorbol treatment. This time course of down-regulation and the presence of phorbol-insensitive isozymes must be considered in interpreting the effects of phorbol esters on bone remodeling. The results of these studies also provide important information that will aid in elucidating the role of specific PKC isozymes in remodeling.

Despite the fact that a range of hormone treatment strategies were employed, no definitive effect of estrogen or tamoxifen on PKC isozyme expression was found in osteoblasts. The results obtained in the two most recent experiments in UMR-106 cells, which indicate that tamoxifen increases the expression of PKC- β_1 , hold promise, but additional experiments are necessary to examine the reproducibility of the observed effect.

The inability to readily detect an effect of estrogen/tamoxifen on PKC isozyme expression in osteoblasts could be due to several factors. First, the PKC isozymes in osteoblasts may not be regulated by estrogen/tamoxifen. Another possibility is that the PKC isozymes in the osteoclast, not the osteoblast, are regulated by estrogen/tamoxifen; experiments will be performed to explore this possibility. If no definitive hormone responsive PKC isozymes are found in either cell type, this implies that some other molecular mechanism underlies the bone-sparing effects of these agents. Second, the hormone treatment strategies employed may not have been appropriate for determining an effect on isozyme expression (*e.g.* longer treatment periods may be necessary to elicit an effect). Third, the variable osteoblastic expression of PKC- δ , which has been shown to be regulated by estrogen in other tissues, may have implications regarding these studies. Fourth, the differentiated state of the osteoblast may influence its hormone responsiveness; experiments with human osteoblastic cell lines and primary mouse osteoblasts are underway as a means of addressing this issue.

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APPENDIX 1: Figures

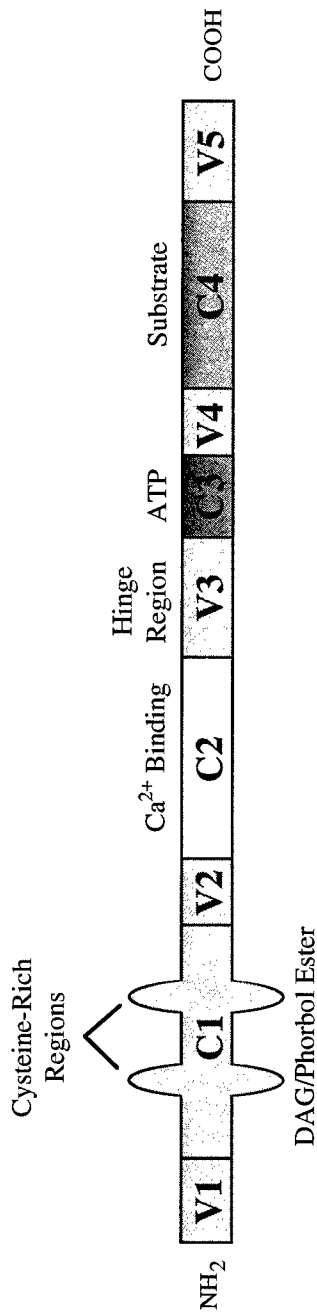
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Regulatory Domain

Catalytic Domain

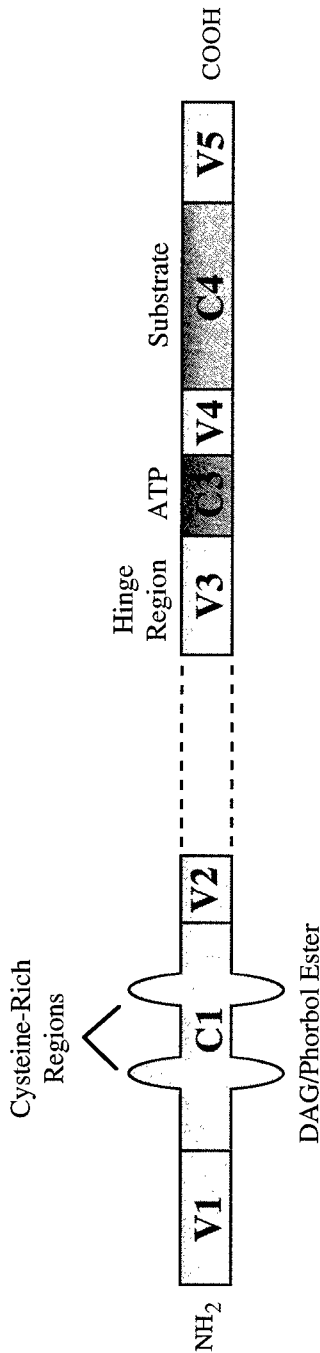
Conventional PKCs

- α
- β_I
- β_{II}
- γ



Novel PKCs

- δ
- ϵ
- η
- θ



Atypical PKCs

- ζ
- ι/λ

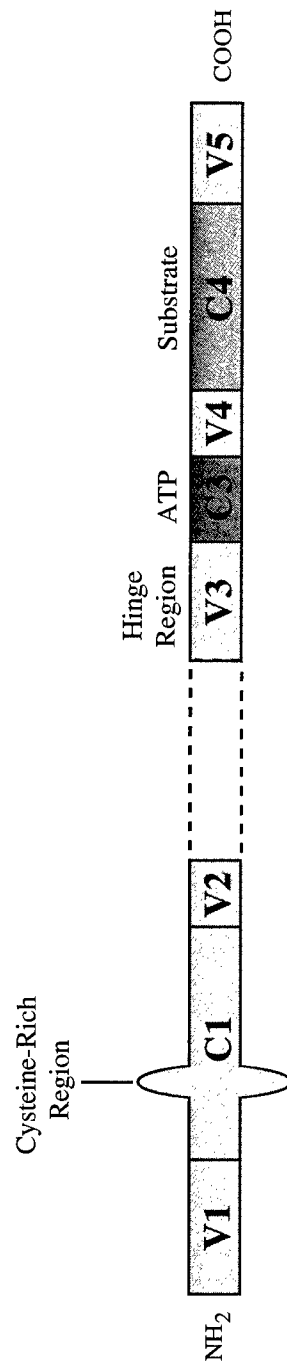


Figure 1. The protein kinase C enzyme family. Conserved (C) and variable (V) domains are indicated as are the binding sites for diacylglycerol (DAG), phorbol ester, calcium, ATP, and substrate.

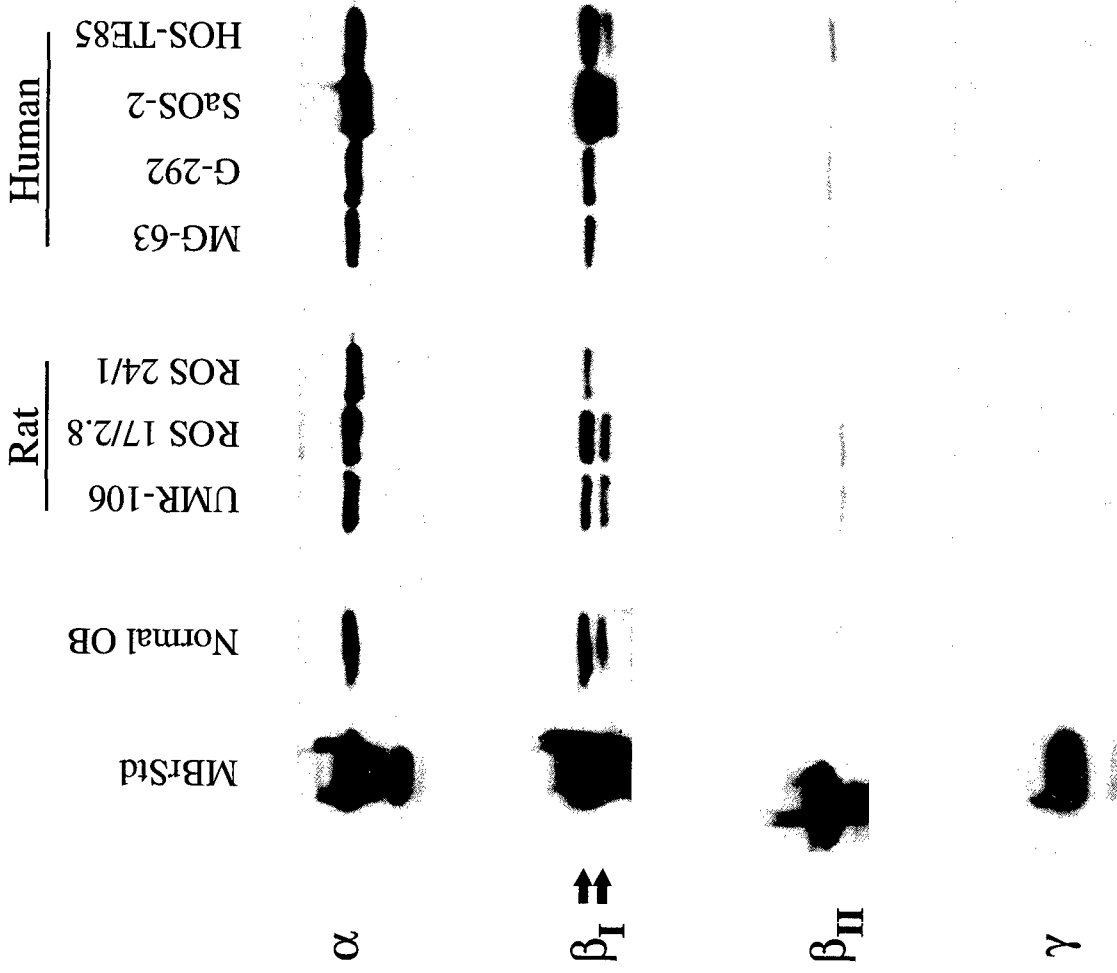


Figure 2. Expression of conventional PKC isozymes in osteoblasts. Whole cell lysates from normal mouse osteoblasts (Normal OB) and human osteoblastic osteosarcoma cell lines were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. A homogenate prepared from a whole mouse brain (MBrStd) was included as a positive control.

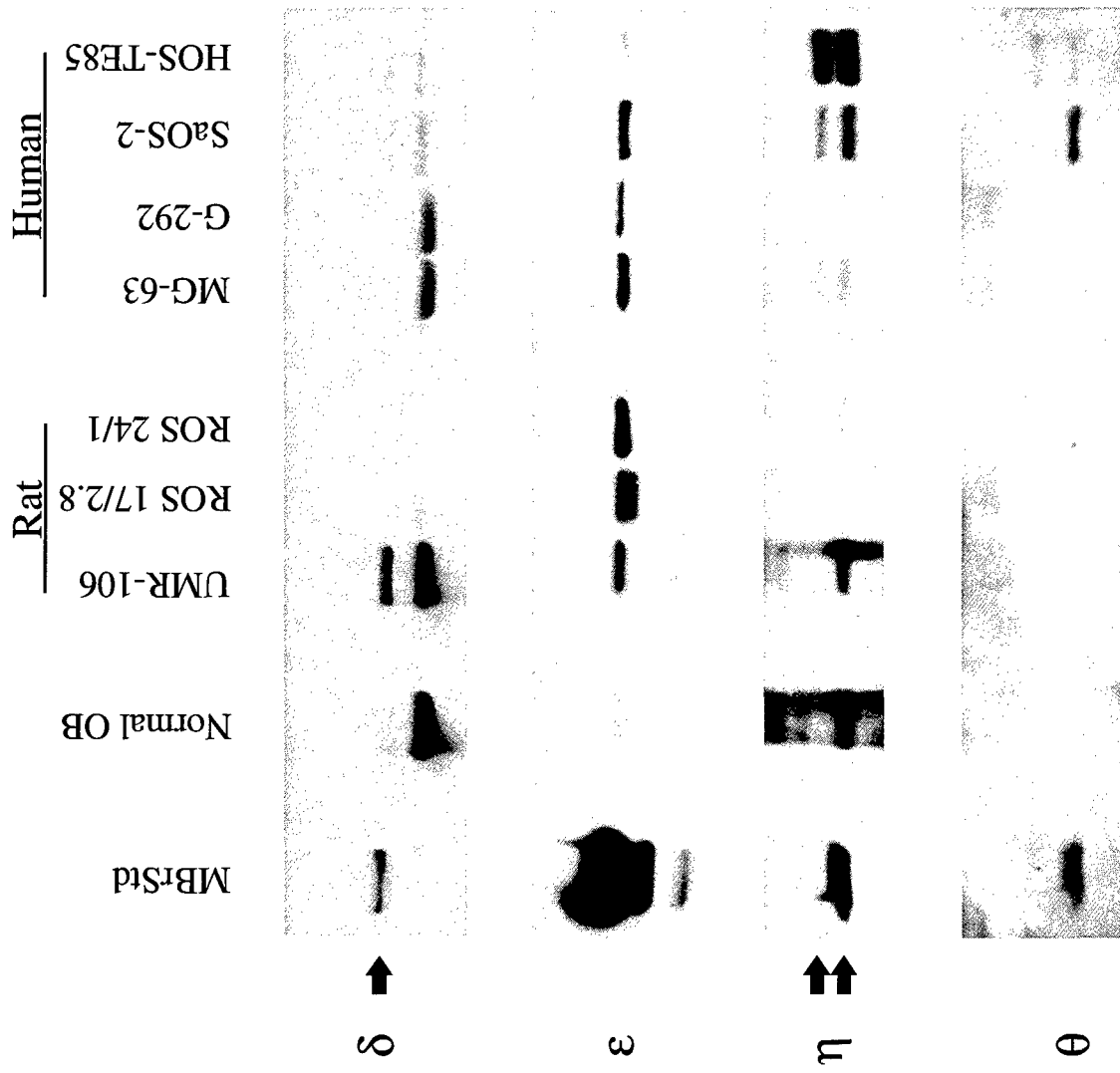


Figure 3. Expression of novel PKC isozymes in osteoblasts. Whole cell lysates from normal mouse osteoblasts (Normal OB) and rat and human osteoblastic osteosarcoma cell lines were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies.

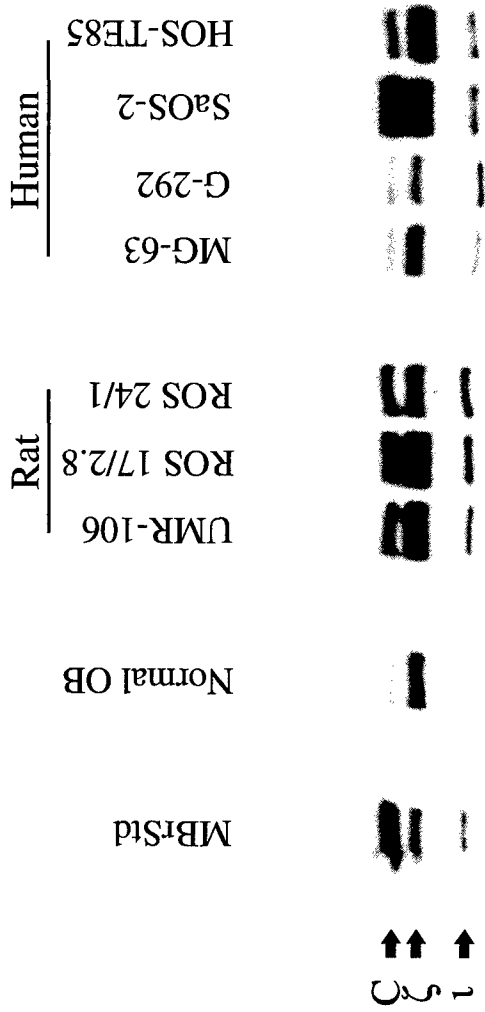


Figure 4. Expression of atypical PKC isozymes in osteoblasts. Whole cell lysates from normal mouse osteoblasts (Normal OB) and rat and human osteoblastic osteosarcoma cell lines were subjected to Western blot analysis with the anti-PKC- ζ antibody; this antibody cross-reacts with PKC- $1/\lambda$ and a conventional PKC isozyme (C) (PKC- $1/\lambda$ is referred to simply as PKC- 1 in this and subsequent figures).

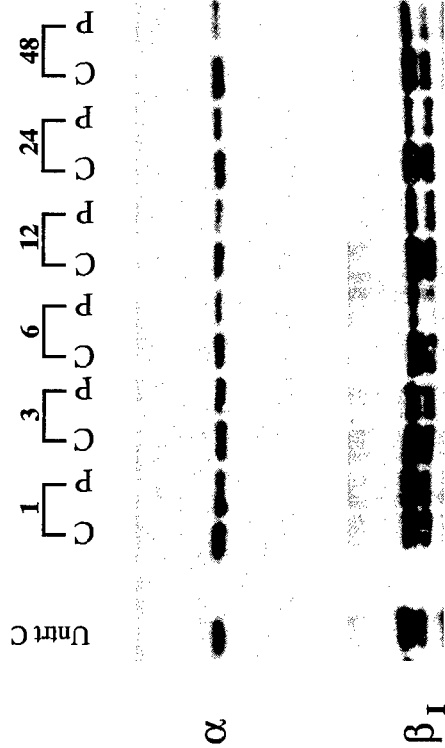
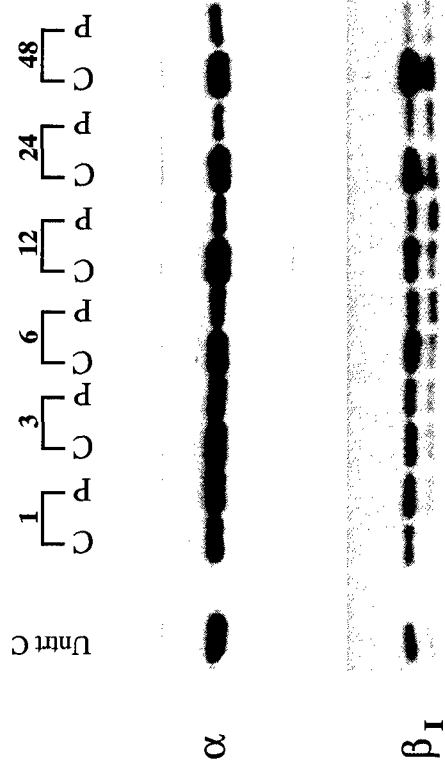
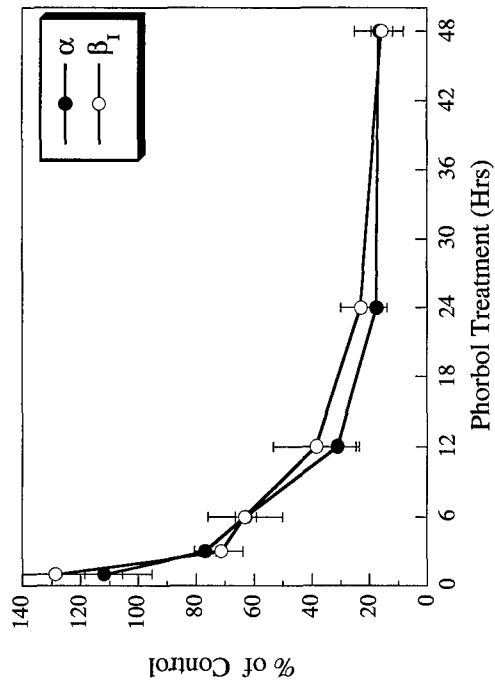
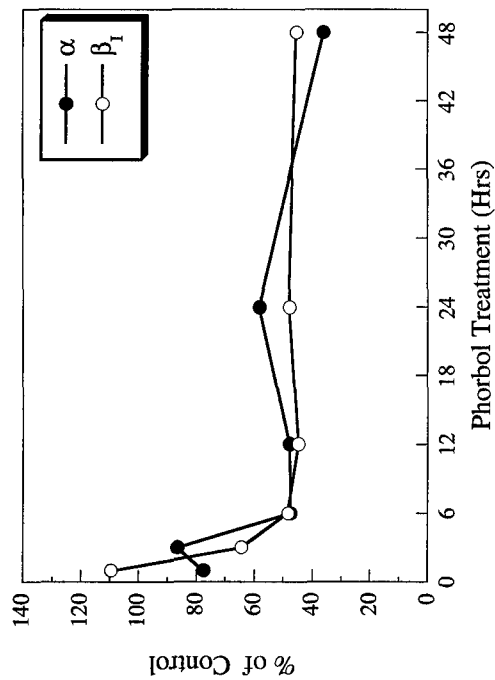
A**Normal Mouse Osteoblasts****UMR-106 Osteoblastic Cell Line****B**

Figure 5. Time course of phorbol ester-induced down-regulation of conventional PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle (C) or 1 μ M PDB (P); untreated control (Untrt C). Whole cell lysates were subjected to Western blot analysis. **A.** Blots with anti-PKC- α and - β_1 antibodies. **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blots shown. The plot for the UMR-106 cells is the mean \pm standard error (SE) of three experiments; a representative blot for each isozyme is shown.

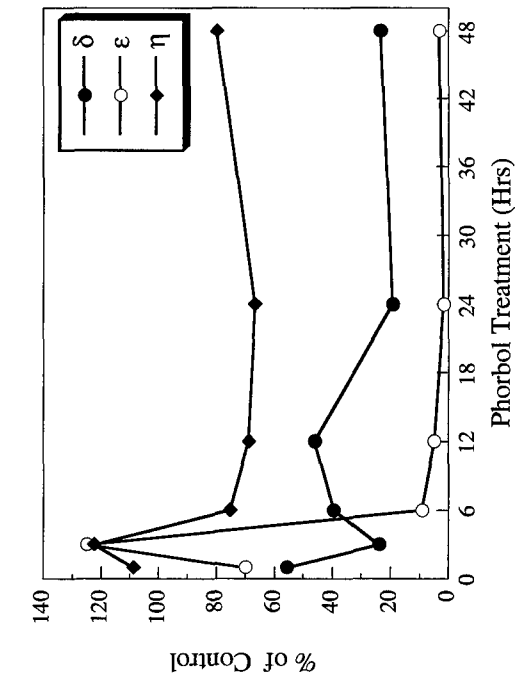
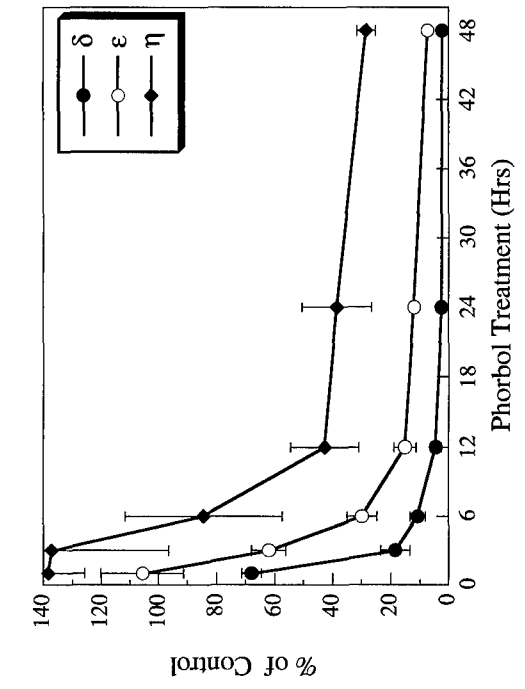
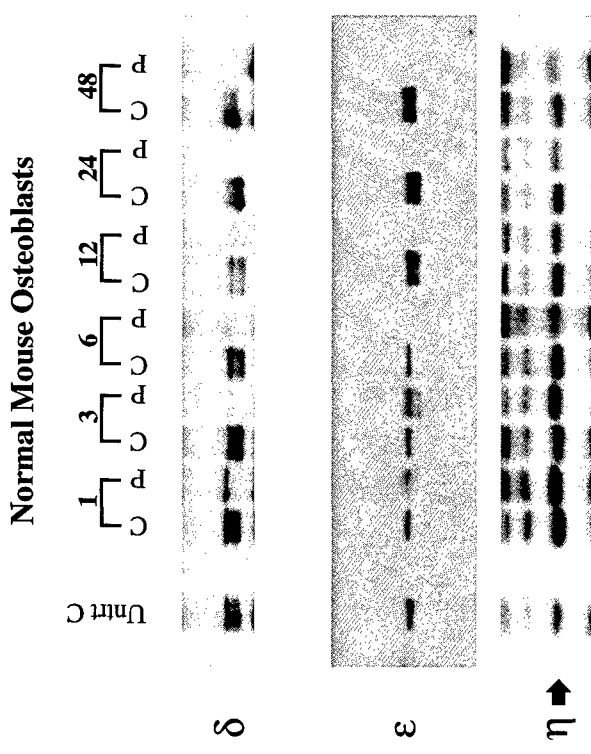
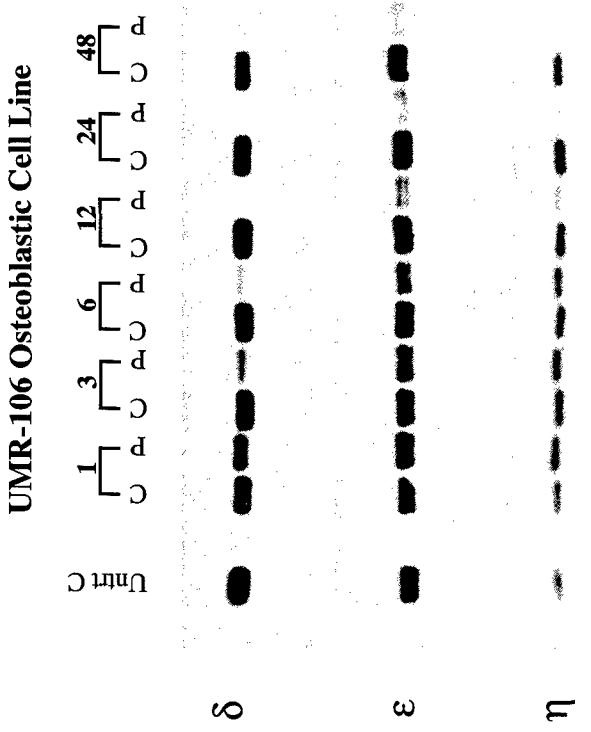


Figure 6. Time course of phorbol ester-induced down-regulation of novel PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle (C) or 1 μ M PDB (P); untreated control (Untrt C). Whole cell lysates were subjected to Western blot analysis. **A.** Blots with anti-PKC- δ , - ϵ , and - η antibodies. **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blots shown. The plot for the UMR-106 cells is the mean \pm SE of three experiments; a representative blot for each isozyme is shown.

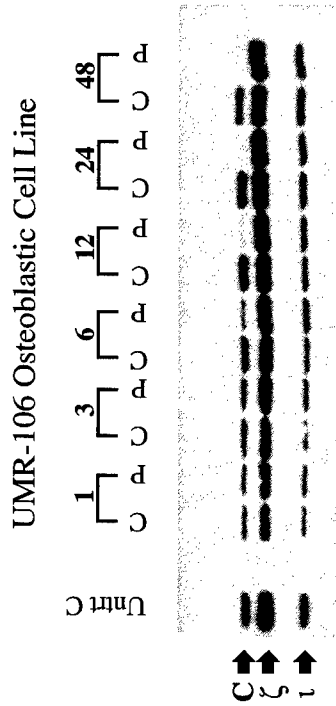
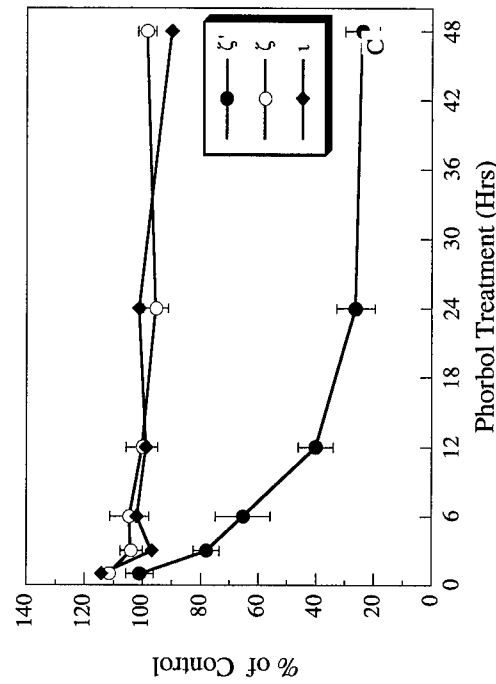
A**B**

Figure 7. Phorbol ester treatment fails to down-regulate atypical PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle (C) or 1 μ M PDB (P); untreated control (Unt C). Whole cell lysates were subjected to Western blot analysis. **A.** Blots with the anti-PKC- ζ antibody, which cross-reacts with PKC- ζ and a conventional PKC isozyme (C). **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blots shown. The plot for the UMR-106 cells is the mean \pm SE of three experiments; a representative blot for each isozyme is shown.

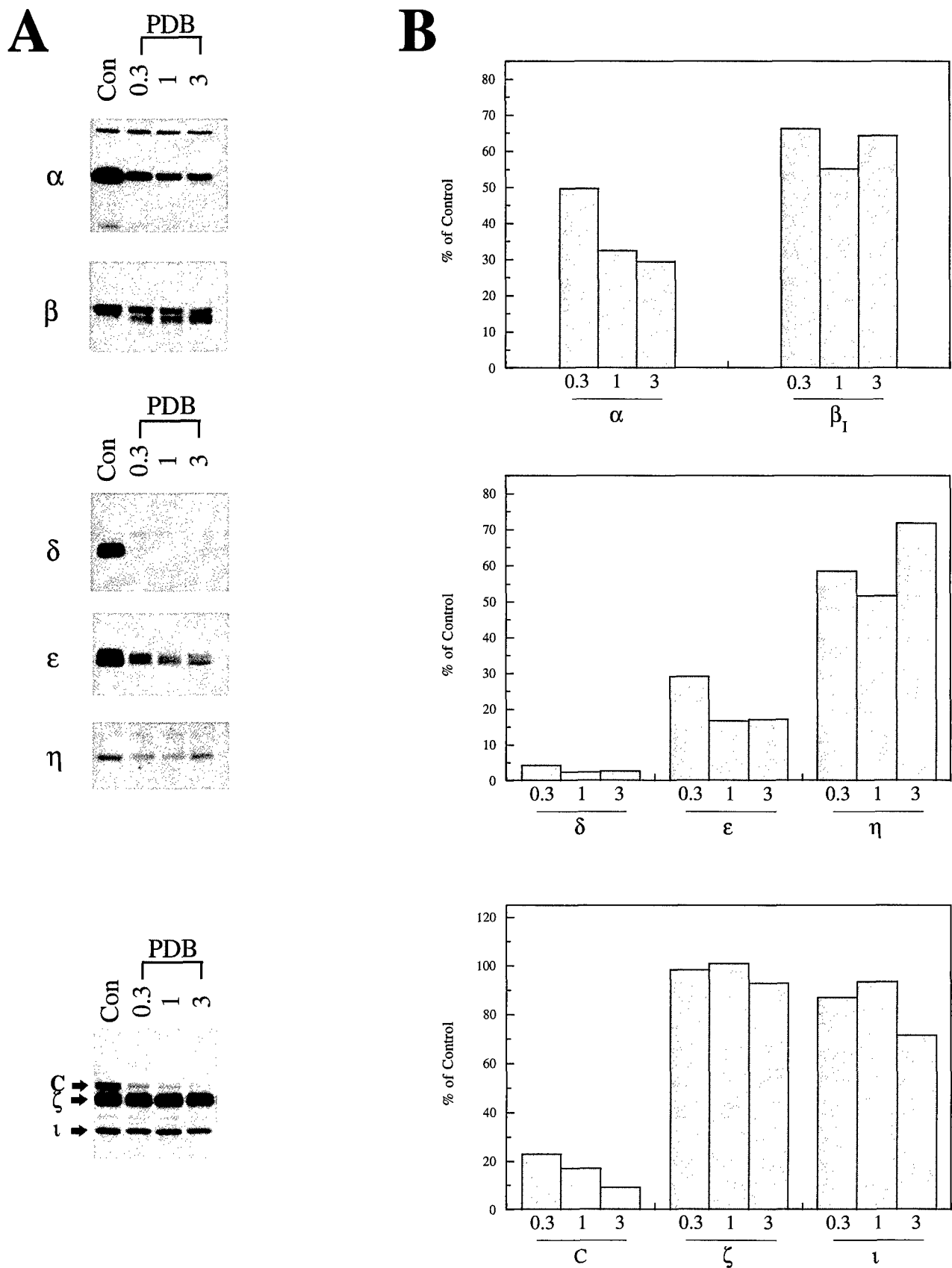


Figure 8. Dose response for PDB-induced down-regulation of PKC isozymes in osteoblasts. UMR-106 cells were treated with vehicle (Con) or 0.3 μ M, 1 μ M, or 3 μ M PDB for 48 h. Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Blots for the conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in A.

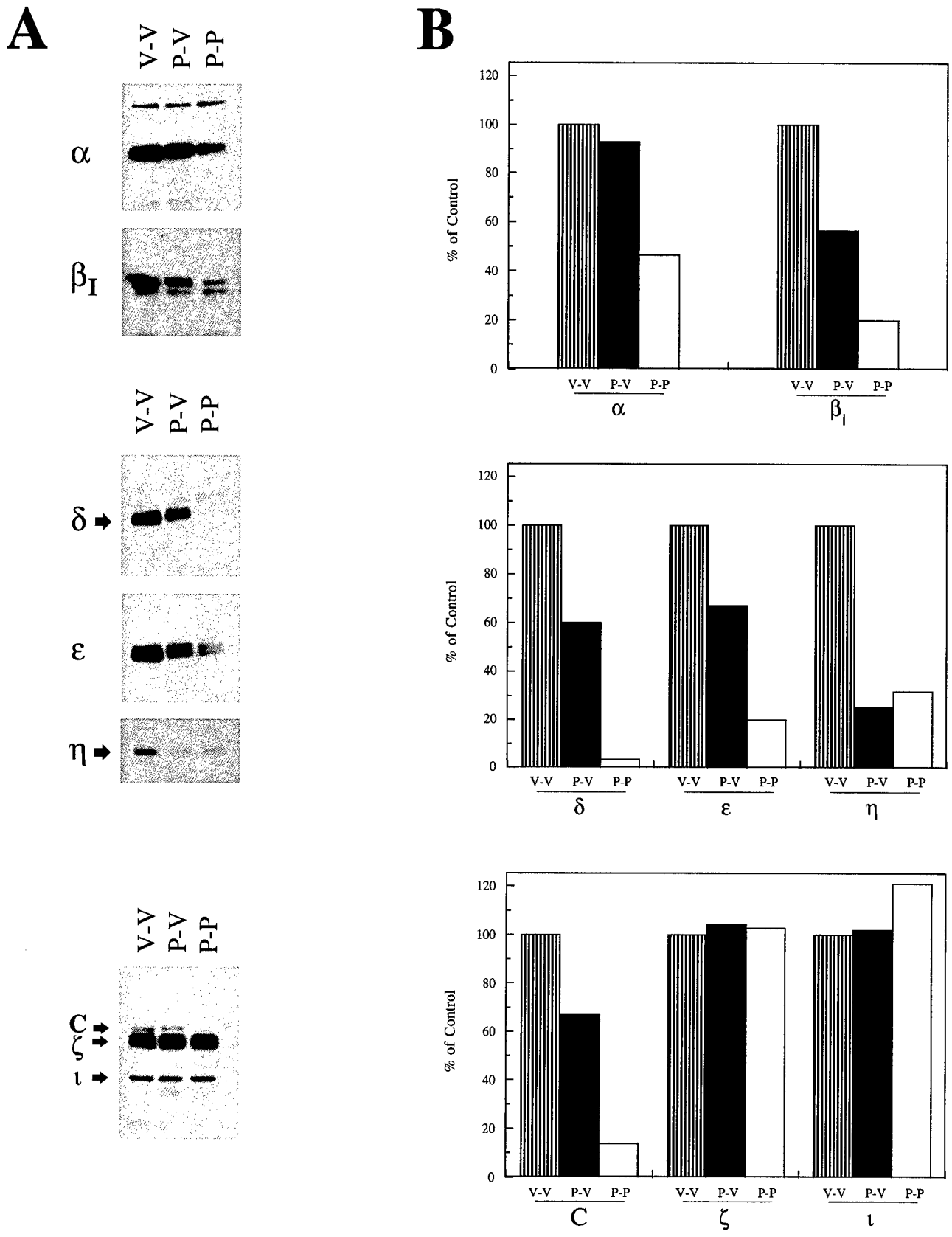
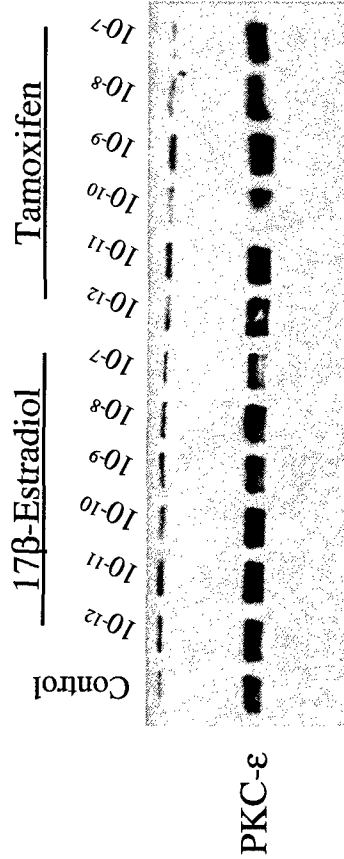
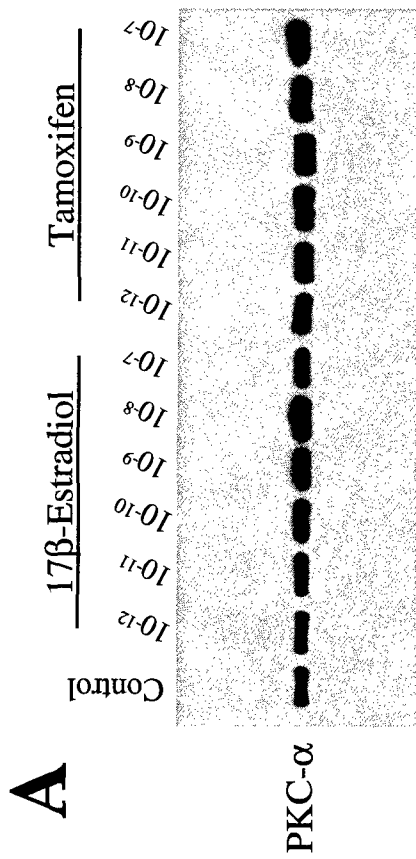


Figure 9. Phorbol ester-sensitive PKC isozymes recover following withdrawal of the phorbol ester. UMR-106 cells were treated for 48 h, with a medium change at 24 h. The cells were treated with vehicle for 48 h (V-V), 1 μ M PDB for 48 h (P-P), or 1 μ M PDB for the initial 24 h period and vehicle for the final 24 h (P-V). Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Blots for the conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in A.



B

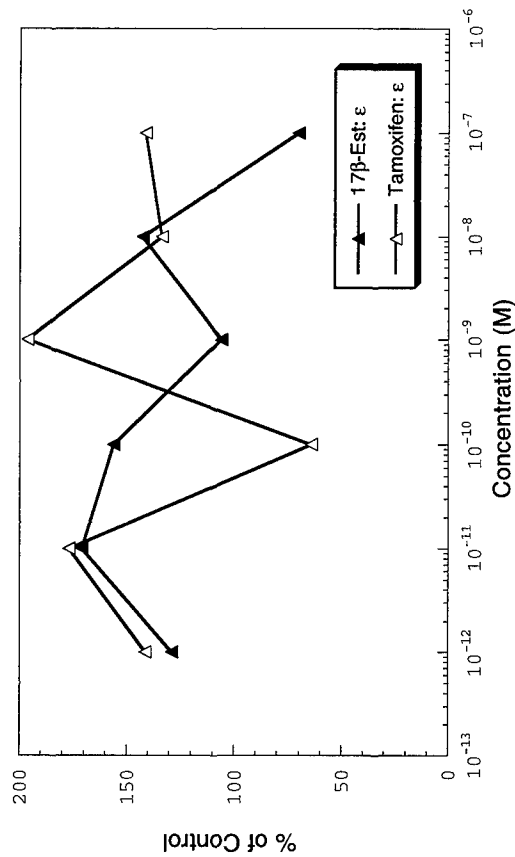
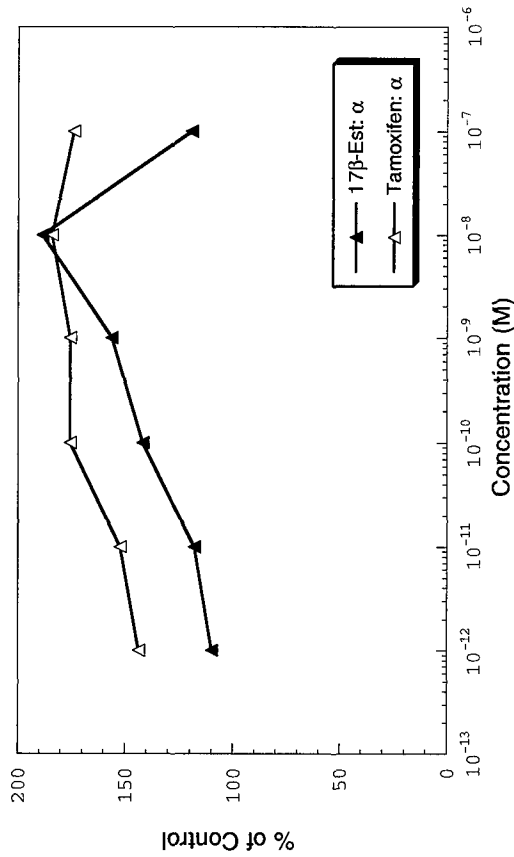


Figure 10. 24 h treatment of confluent UMR-106 cells with vehicle (Control), 17 β -estradiol, or 4-OH-tamoxifen (10⁻¹² to 10⁻⁷ M). Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Western blots for PKC- α and PKC- ϵ . **B.** Densitometric analysis of the blots shown in **A.**

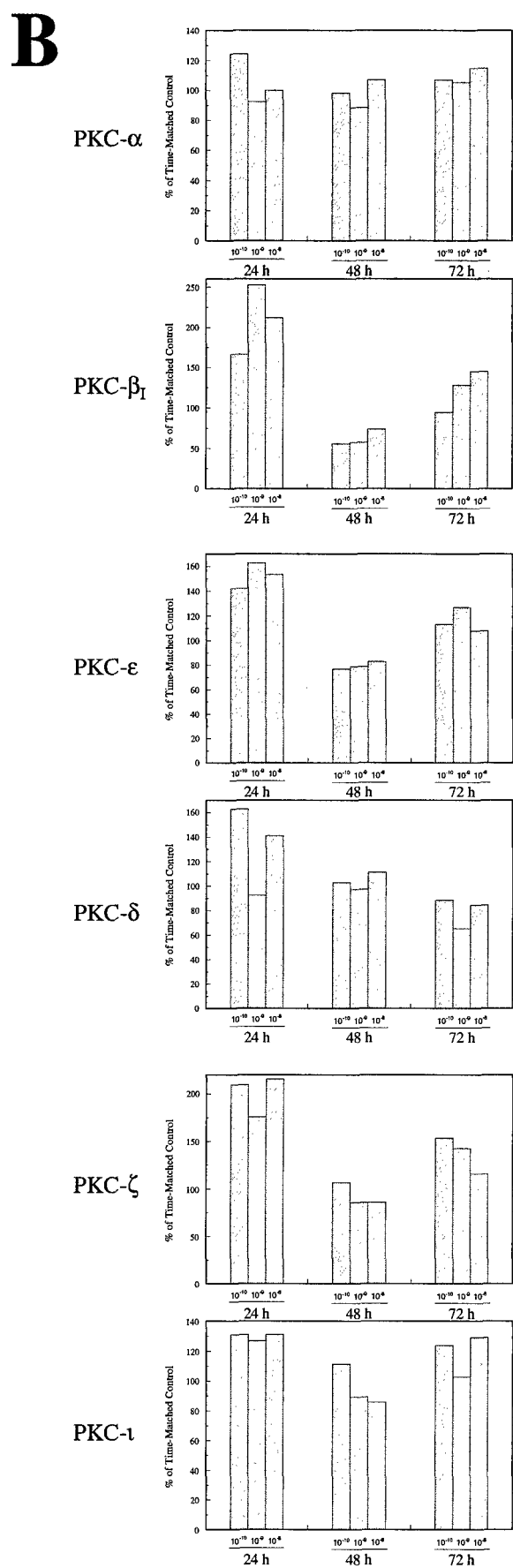
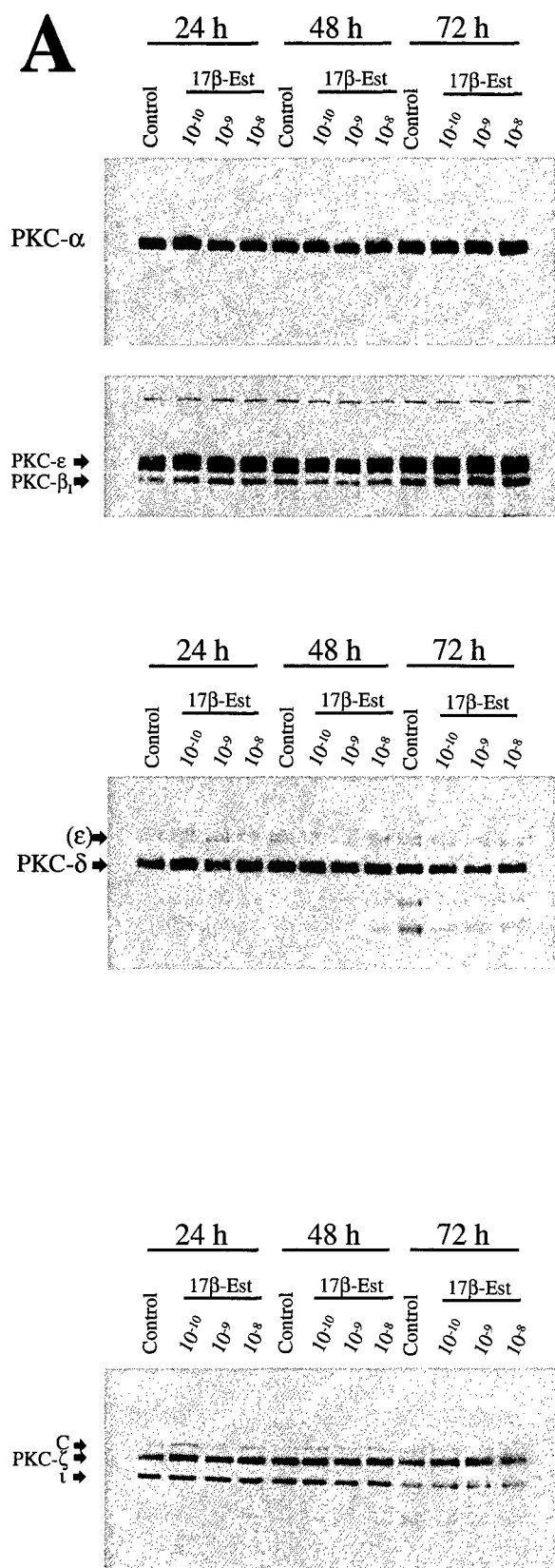


Figure 11. 24, 48, or 72 h treatment of confluent UMR-106 cells with 17 β -estradiol (10⁻¹⁰ to 10⁻⁸ M). Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Blots for the conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in A.

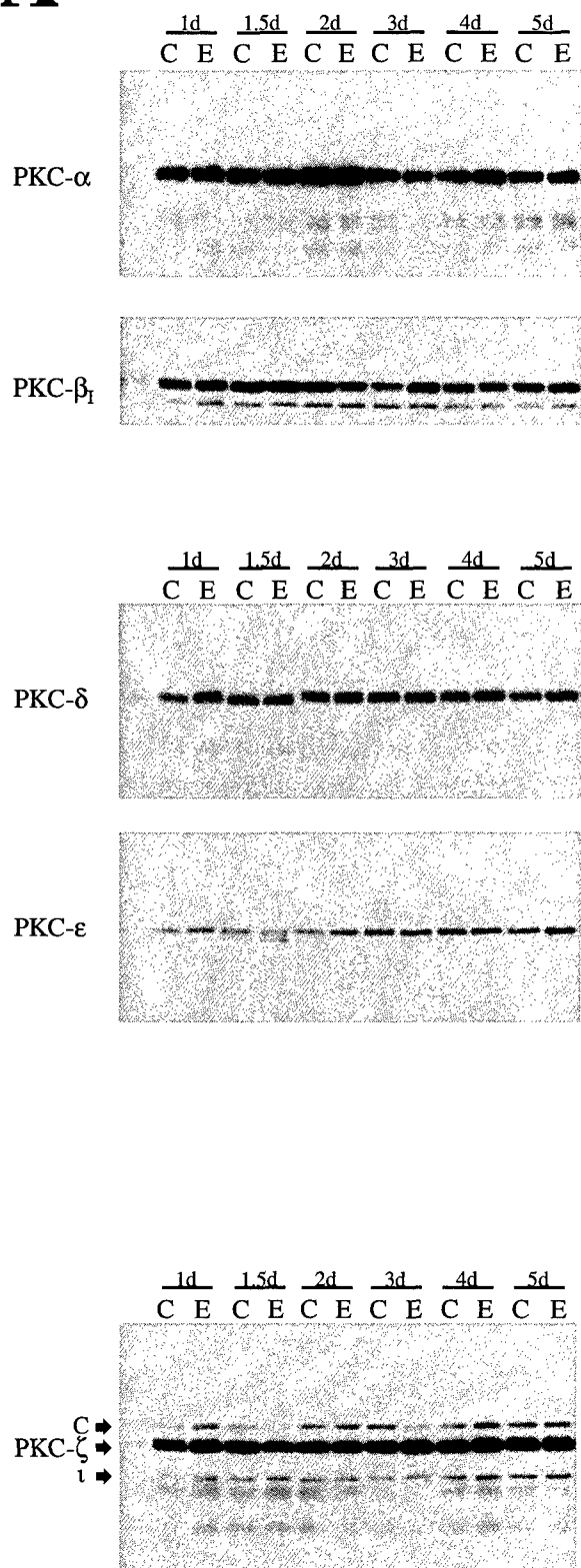
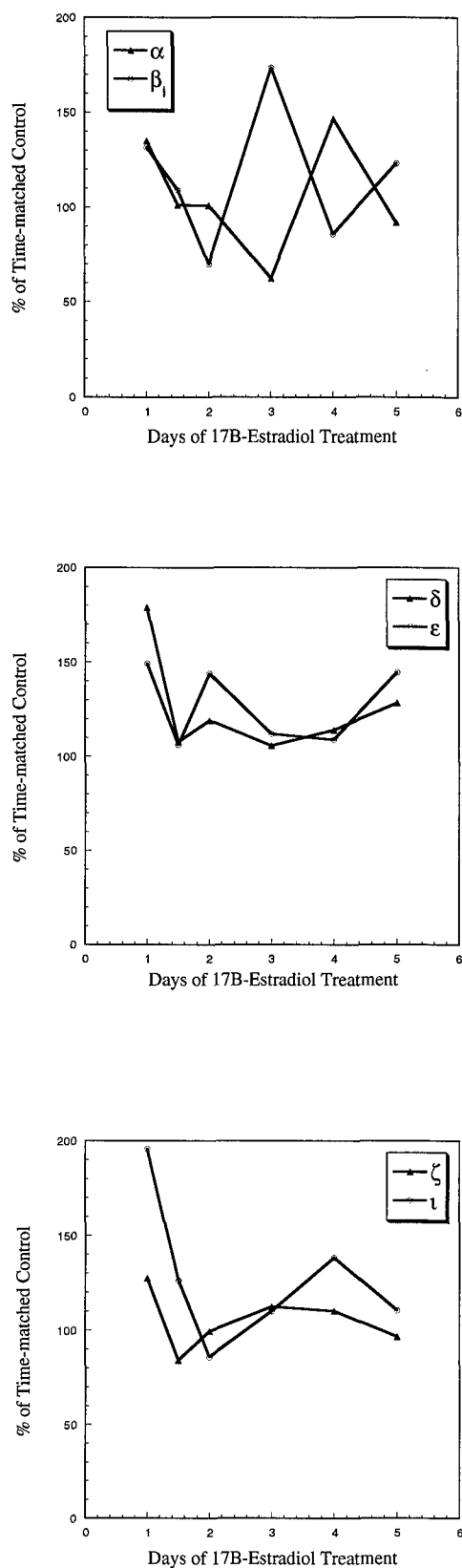
A**B**

Figure 12. 1-5 Day treatment of subconfluent UMR-106 cells with vehicle (C) or 17β-estradiol (E; 10⁻⁹ M). Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Blots for conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in A.

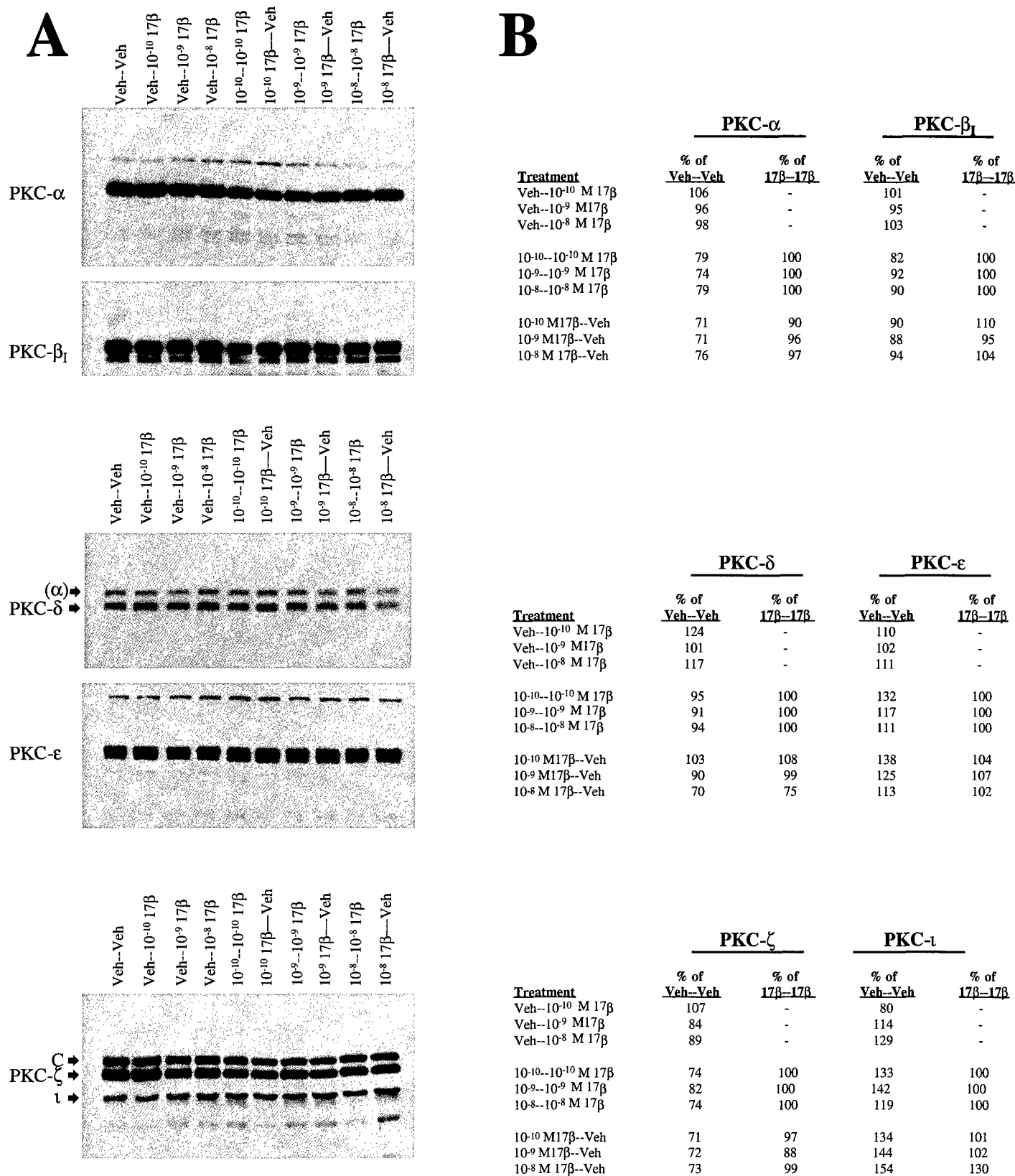


Figure 13. Determination of the effect of estrogen withdrawal on PKC isozyme expression in UMR-106 cells. Cells were treated for 6 days, as follows: 6 day vehicle treatment (Veh-Veh), 3 day vehicle treatment then 3 day 17 β -estradiol treatment (Veh-17 β ; 10⁻¹⁰ to 10⁻⁸ M), 6 day 17 β -estradiol treatment (17 β -17 β), or 3 day 17 β -estradiol treatment then 3 day vehicle treatment (17 β -Veh). **A.** Western blots for the conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in **A.**

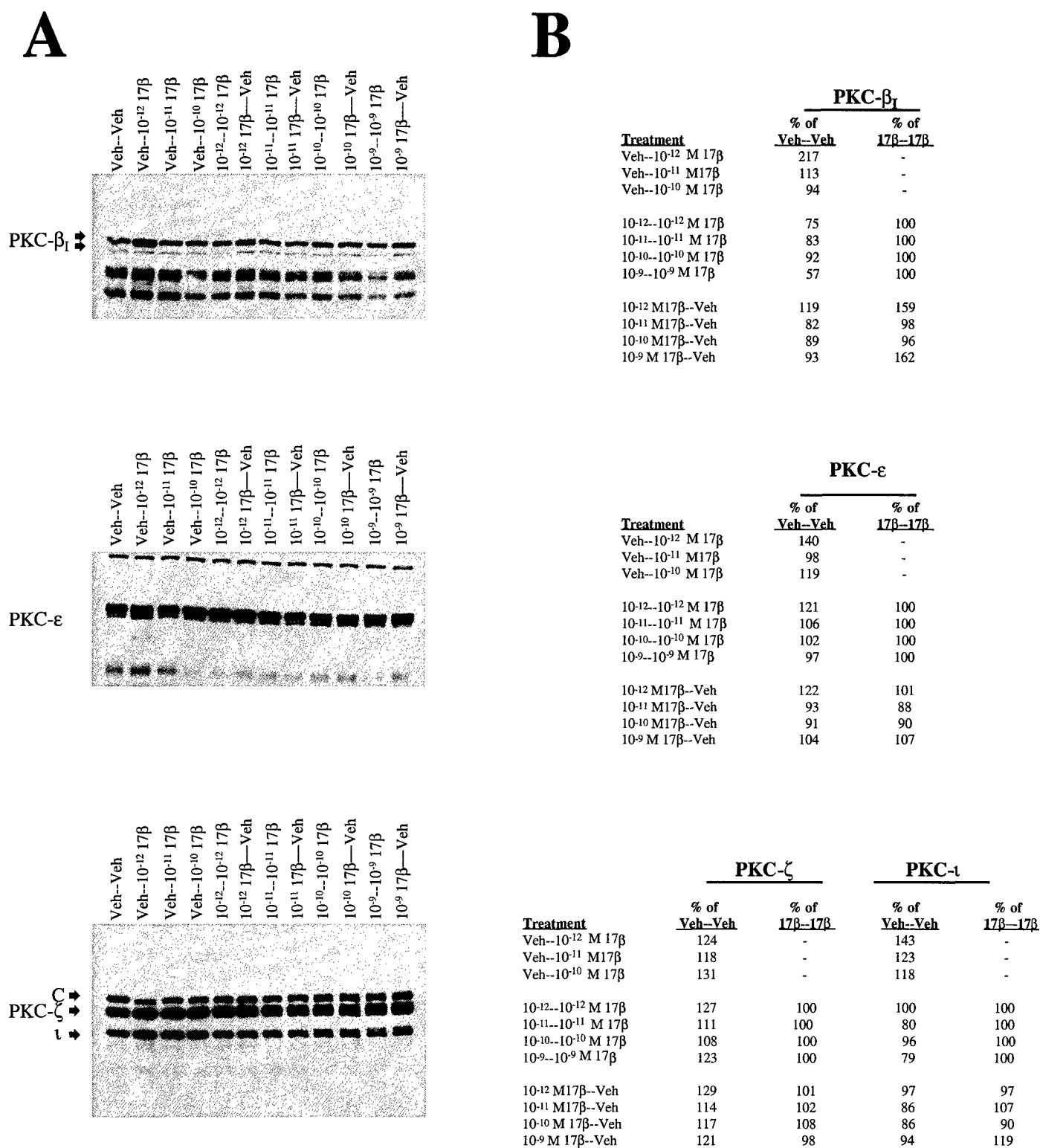


Figure 14. Determination of the effect of estrogen withdrawal on PKC isozyme expression in UMR-106 cells. Cells were treated for 6 days, as follows: 6 day vehicle treatment (Veh-Veh), 3 day vehicle treatment then 3 day 17β-estradiol treatment (Veh-17β; 10⁻¹² to 10⁻¹⁰ M), 6 day 17β-estradiol treatment (17β-17β; 10⁻¹² to 10⁻⁹ M), or 3 day 17β-estradiol treatment then 3 day vehicle treatment (17β-Veh). **A.** Western blots for PKC-β₁, -ε, -ζ, and -ι. **B.** Densitometric analysis of the blots shown in A.

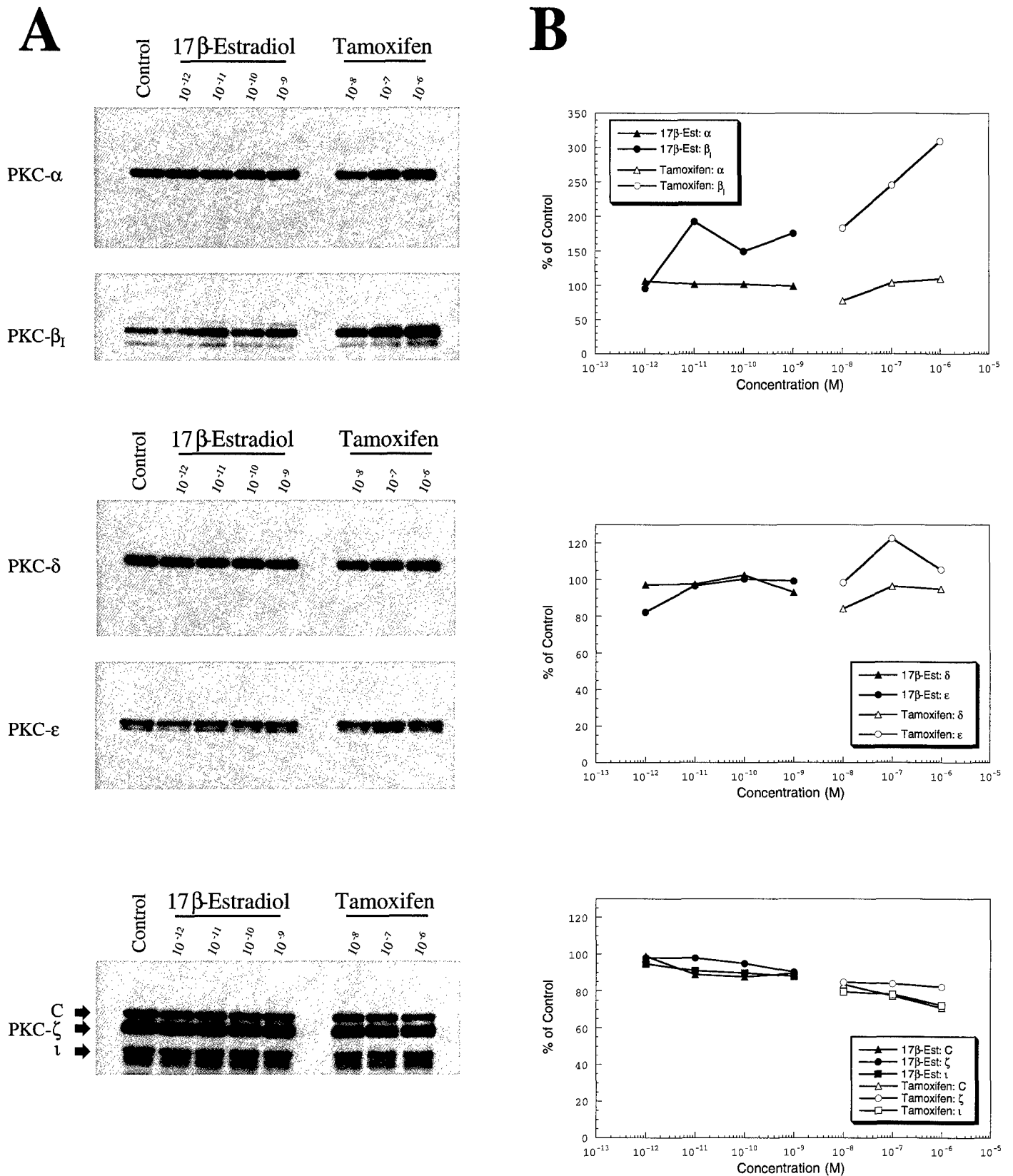


Figure 15. 7 Day treatment of subconfluent UMR-106 cells with vehicle (control), 17 β -estradiol (10^{-12} to 10^{-9} M), or 4-OH-tamoxifen (10^{-8} to 10^{-6} M). Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Western blots for conventional, novel, and atypical isozymes. **B.** Densitometric analysis of the blots shown in **A.**

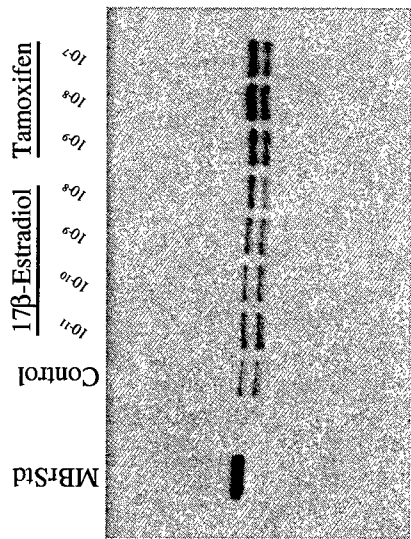
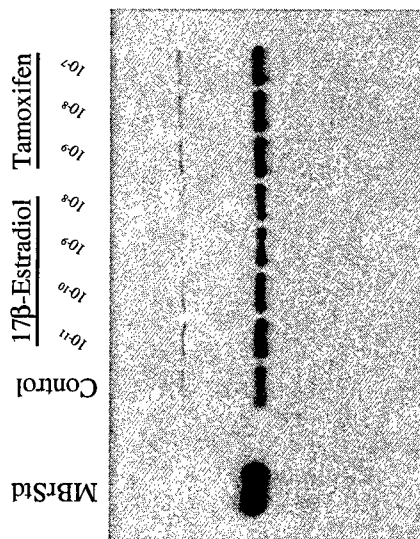
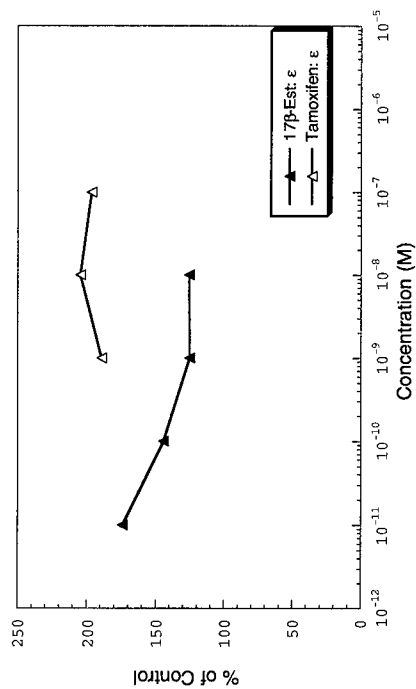
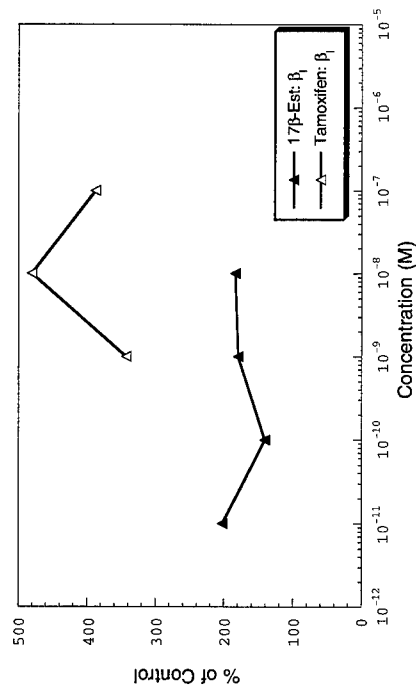
APKC- β_1 PKC- ϵ **B**

Figure 16. 3 Day treatment of subconfluent UMR-106 cells with vehicle (control), 17 β -estradiol (10⁻¹¹ to 10⁻⁸ M), or 4-OH-tamoxifen (10⁻⁹ to 10⁻⁷ M). Treatments were carried out in phenol red-free medium supplemented with ITS. Whole cell lysates were subjected to Western blot analysis with isozyme-specific anti-PKC antibodies. **A.** Western blots for PKC- β_1 and PKC- ϵ . **B.** Densitometric analysis of blots shown in **A.**

APPENDIX 2: Manuscript

A manuscript entitled "Expression and phorbol ester-induced down-regulation of protein kinase C isozymes in osteoblasts" has been accepted for publication in the Journal of Bone and Mineral Research. A copy of this manuscript follows.

Because this paper has not yet been published, please limit the distribution of this document.

EXPRESSION AND PHORBOL ESTER-INDUCED DOWN-REGULATION OF PROTEIN
KINASE C ISOZYMES IN OSTEOBLASTS

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Running title: PKC Isozymes in Osteoblasts

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10:A680).

ABSTRACT

The protein kinase C (PKC) enzyme family consists of at least 11 isozymes in 3 classes, with characteristic tissue distributions. Phorbol esters activate and ultimately down-regulate phorbol-sensitive isozymes. PKC is a signal transducer in bone, and phorbol esters influence bone resorption. Little is known about specific PKC isozymes in this tissue, however. We describe here the expression and phorbol ester-induced down-regulation of PKC isozymes in osteoblasts. Normal mouse osteoblasts and seven osteoblastic cell lines (rat UMR-106, ROS 17/2.8, ROS 24/1, and human MG-63, G-292, SaOS-2, HOS-TE85) were screened for isozyme expression by Western immunoblotting using isozyme-specific anti-PKC antibodies. The conventional α and β_I isozymes, but not γ , were present in each of the osteoblasts examined; PKC- β_{II} was detectable in all but the ROS 24/1 line. PKC- ϵ was expressed in all osteoblasts screened, but other novel PKCs, δ , η , and θ , were detectable only in select lines. The atypical ζ and ι/λ PKCs were in all osteoblasts examined. To determine the sensitivity of the isozymes to prolonged phorbol ester treatment, normal osteoblasts and the UMR-106 cell line were treated with vehicle or 1 μ M phorbol 12, 13-dibutyrate (PDB) for 1, 3, 6, 12, 24, or 48 h, and Western blot analysis was performed. Normal and UMR-106 cells showed similar phorbol sensitivities; conventional (α , β_I) and novel (δ , ϵ , η) isozymes were down-regulated by prolonged phorbol treatment but atypical isozymes were not. Down-regulation of all sensitive PKCs was detectable within 6 h of phorbol treatment; the novel δ and ϵ isozymes, however, showed more rapid and dramatic down-regulation than conventional isozymes. The observed down-regulation was dose-dependent (0.3-3 μ M) and specific; 48 h treatment with the inactive phorbol, 4 α -phorbol 12,13-didecanoate (4 α -PDD), failed to down-regulate PDB-sensitive isozymes. The phorbol-induced down-regulation was also

reversible; 24 h after withdrawing PDB, all phorbol-sensitive isozymes, except PKC- η , had recovered at least partially. These studies, which are the first to thoroughly characterize PKC isozyme expression in osteoblastic cells, demonstrate that osteoblasts have a characteristic PKC isozyme profile, including both phorbol ester-sensitive and -insensitive isozymes. The time course of down-regulation and the presence of phorbol-insensitive PKCs must be considered in interpreting the effects of phorbol esters on bone remodeling.

Key words: Osteoblasts, Protein kinase C isozymes, Phorbol ester, Down-regulation, Western immunoblotting

INTRODUCTION

The protein kinase C (PKC) family of serine/threonine kinases consists of at least 11 isozymes with unique tissue distributions and substrate specificities⁽¹⁻⁸⁾. These isozymes comprise at least three classes, termed the conventional, novel, and atypical isozymes. The PKC isozymes in a given class have similar activation requirements due to common structural features within each class. The conventional isozymes, PKC- α , - β _I, - β _{II}, and - γ , require phosphatidylserine (PS), diacylglycerol (DAG), and calcium (Ca^{2+}) for activation⁽¹⁻³⁾. The novel isozymes, PKC- δ , - ϵ , - η , and - θ also require PS and DAG for activation, but are Ca^{2+} -independent⁽¹⁻⁶⁾. The atypical isozymes, PKC- ζ and - ι/λ , require PS but are both Ca^{2+} - and DAG-independent^(1-3,7,8).

The common structural features of a given class of PKCs also confer a similar sensitivity of the isozymes to phorbol esters. Phorbol esters act as DAG analogs⁽⁹⁾; therefore, the conventional and novel PKC isozymes, which bind DAG, also bind and are responsive to phorbol esters⁽¹⁻⁶⁾. In contrast, the atypical isozymes, which lack one of the two cysteine-rich zinc finger motifs necessary to bind DAG, also fail to bind phorbol esters and are generally phorbol-insensitive^(7,8,10-13). Phorbol ester-sensitive PKC isozymes are activated by acute phorbol treatment⁽⁹⁾ but down-regulated with more prolonged exposures⁽¹⁴⁾.

PKC has been implicated in the signalling events associated with numerous cellular responses, including bone resorption⁽¹⁵⁻²⁴⁾. The role of PKC in bone remodeling is not clear, however. PKC activity is increased in particulate fractions from parathyroid hormone (PTH)-treated bones or bone cells⁽¹⁵⁻¹⁸⁾, and antagonists of PKC prevent PTH- and calcitriol-stimulated

resorption^(19,20). In different studies, phorbol esters have been found to stimulate bone resorption⁽²¹⁻²³⁾ and to inhibit resorptive responses to PTH⁽²⁴⁾.

Little is known about specific PKC isozymes in bone tissue. We describe here the expression and the phorbol ester-induced down-regulation of PKC isozymes in osteoblastic cells, which are known to be important in both bone formation and resorption. Normal mouse osteoblasts and seven osteoblastic cell lines were screened for isozyme expression by Western immunoblotting using isozyme-specific anti-PKC antibodies. Western blotting was also employed to characterize the phorbol ester sensitivity of the osteoblastic isozymes. The results of these studies provide important information that will aid in elucidating the role of PKC in bone remodeling.

MATERIALS AND METHODS

Cell Culture

Normal mouse osteoblasts were isolated enzymatically under sterile conditions from neonatal (5-6 day) mouse calvaria using a method described previously⁽²⁵⁾. This method was modified, however, to include six 20 min incubations with the enzyme. After isolation, osteoblasts were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated horse serum, 100 U/ml K-penicillin G, and 10 U/ml Na-heparin, and added to culture dishes. The cells were allowed to attach overnight at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed the following day to remove unattached cells.

UMR-106 rat osteoblastic osteosarcoma cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in 75 cm² cell culture flasks at 37°C in a humidified 5% CO₂ atmosphere in DMEM supplemented with 15% heat-inactivated horse serum and 100 U/ml K-penicillin G. UMR-106 cells between the 6th and 17th passage were used for experiments. The ROS 17/2.8 and ROS 24/1 rat osteoblastic cell lines were provided by Dr. S.B. Rodan of Merck and were cultured as previously described⁽²⁶⁾. Rat cells were passaged every 5-7 days with medium changes every 3 days. The four human osteoblastic cell lines used, MG-63, G-292, SaOS-2, and HOS-TE85, (American Type Culture Collection) were cultured under conditions recommended by the supplier.

Experimental protocols

A. Isozyme expression in osteoblastic cells

The normal osteoblasts and the osteoblastic cell lines were approximately 40-50% confluent when harvested.

B. Determination of the sensitivity of PKC isozymes to prolonged phorbol ester treatment

Normal mouse osteoblasts and UMR-106 rat osteoblastic cells were selected for phorbol ester time course studies. After isolation, normal mouse osteoblasts were seeded in 10 cm dishes in 10 ml of culture medium. The medium was changed on both the first and second days after isolation. On the third day after isolation, treatments were initiated. Phorbol 12, 13-dibutyrate (PDB; Sigma Chemical Company, St. Louis, MO, U.S.A.) or an equal volume of vehicle (dimethyl sulfoxide (DMSO); Sigma) was added directly to the culture medium to give a final PDB concentration of 1 μ M; the contents of the dishes were mixed by swirling. Cells were treated with PDB or vehicle for 1, 3, 6, 12, 24, or 48 h.

Time course experiments with the UMR-106 cells were carried out as described for the normal mouse osteoblasts except that phorbol ester or vehicle treatments were initiated the day after the UMR-106 cells were seeded (1.5×10^6 cells/dish) into 10 cm dishes.

Dose response and withdrawal experiments with PDB were carried out with UMR-106 cells, as were experiments with the inactive phorbol, 4 α -phorbol 12, 13-didecanoate (4 α -PDD; Sigma). For each of these studies, UMR-106 cells were seeded in 10 cm dishes (1.5×10^6 cells/dish), and treatments initiated the following day. For dose response studies, cells were treated for 24 or 48 h with vehicle or 0.3 μ M, 1 μ M, or 3 μ M PDB. Withdrawal experiments involved 3 dishes of cells; one dish was treated with vehicle for 48 h, the second with 1 μ M PDB for 48 h, and the third with 1 μ M PDB for the first 24 h and vehicle for the final 24 h. After the initial 24 h period, all dishes were handled in the same way; that is, the medium was aspirated, the cells were

washed 3X with culture medium, and fresh treatments were added for an additional 24 h. For the 4 α -PDD studies, cells were treated with either vehicle or 1 μ M 4 α -PDD for 48 h.

Western immunoblotting

The isozyme-specific anti-PKC antibodies utilized for Western blotting (rabbit polyclonal antibodies raised against C-terminal isozyme peptides) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Cells were harvested for Western blotting according to the method suggested in the research applications supplement received with the antibodies; this method is described below.

Cells were removed from culture dishes by scraping in RIPA buffer (phosphate buffered saline (PBS), 1% Nonidet-P40 (NP40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.15-0.3 TIU/ml aprotinin, 1 mM sodium orthovanadate). The cell suspension was transferred to a 1.5 ml microfuge tube using a syringe fitted with a 21 gauge needle. The dish was washed once with additional RIPA buffer, and this was combined with the first lysate. The total volume of lysate was then passed through a 21 gauge needle approximately 15X to shear the DNA. After a 30 min incubation on ice, additional PMSF (0.1 mg/ml) was added to each lysate. The samples were microfuged for 20 min at 4°C, and the resulting supernatant was taken as the whole cell lysate. An aliquot of the supernatant was removed for determination of protein, which was measured by the method of Lowry⁽²⁷⁾. The remaining supernatant was mixed with an equal volume of stop solution (17% glycerol, 8.7% (v/v) 2-mercaptoethanol, 5% SDS, 0.2 M Tris-HCl (pH 6.7), 0.1 mg/ml bromophenol blue) and boiled for 2 min. Samples were stored at -20°C until use.

For immunoblotting⁽²⁸⁾, extracts were subjected to SDS polyacrylamide electrophoresis using 5% stacking gels and 10% separating gels (0.05 amp/gel, 4-5 h) followed by electrophoretic transfer of proteins to nitrocellulose (0.2 μ M, Schleicher & Schuell, Keene, NH, U.S.A.) (30 volts, overnight). After transfer, membranes were blocked in Blotto B (Tris-buffered saline (TBS), 0.05% Tween-20 (T), 1% bovine serum albumin (BSA), 1% Carnation non-fat dry milk) for 1 h at room temperature. Blots were then incubated with isozyme-specific anti-PKC antibodies (1:1000 in Blotto B) for 45 min at room temperature on a rocking platform. Membranes were washed two times in TBS-T and subsequently incubated with goat anti-rabbit, peroxidase-conjugated secondary antibody (Sigma; 1:2000 in Blotto B) for 30 min at room temperature on a rocking platform. The membranes were then washed 3X in TBS-T and once in TBS, and immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL, U.S.A.) using Kodak X-OMAT LS film (Sigma). For the phorbol ester studies, immune complexes were quantified by densitometry using a Bio-Rad Model GS-670 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

In some experiments, membranes were stripped and reprobed with a different anti-PKC antibody. Stripping was done by immersing membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)) for 30 min at 50°C in a shaking water bath. After stripping, the membranes were washed 2X for 10 min in TBS-T. Following these washes, membranes were handled as described above, starting at the point of blocking. Membranes were not stripped more than twice.

In addition to the C-terminal anti-PKC- δ antibody described above, a second PKC- δ antibody was employed to evaluate the expression of this isozyme in the normal osteoblasts and

the osteoblastic cell lines. The second antibody was a mouse monoclonal antibody raised against a peptide derived from the internal portion of the PKC- δ molecule (amino acids 114-289, rat PKC- δ) (Transduction Laboratories, Lexington, KY, U.S.A.). Western blots with this antibody were carried out as described above, except that membranes were blocked with 5% Carnation non-fat dry milk (in PBS-T). Both the primary and the secondary antibody (goat anti-mouse; Sigma) were made up in this blocking buffer (1:1000).

RESULTS

PKC isozyme expression in normal osteoblasts and osteoblastic cell lines

To determine if there is a characteristic pattern of PKC isozyme expression in osteoblasts, the isozyme profiles of eight different osteoblasts/osteoblast-like cell lines were elucidated by Western immunoblotting. The osteoblasts screened included normal neonatal mouse osteoblasts, three rat osteoblastic cell lines (UMR-106, ROS 17/2.8, ROS 24/1), and four human osteoblastic cell lines (MG-63, G-292, SaOS-2, HOS-TE85). These cell lines were selected for study because they are commonly employed osteoblast models; furthermore, screening mouse, rat, and human cells was necessary to permit comparisons across species.

Figure 1A shows the Western blots obtained utilizing antibodies to the conventional PKC isozymes, α , β_I , β_{II} , and γ ; PKC- β_I and β_{II} are derived from a single gene by alternative splicing and differ only in their carboxy-termini (approximately 50 amino acids)⁽¹⁾. PKC- α and - β_I were expressed in each of the osteoblasts examined. Although the reactivity was low, PKC- β_{II} was detectable in all but the ROS 24/1 rat line. The γ isozyme, however, was not detectable in any of the osteoblasts screened.

The molecular weights of the conventional PKC isozymes detected in osteoblasts were consistent with those reported for these isozymes in other tissues⁽³⁾. PKC- α , β_I and β_{II} each migrated with an apparent molecular weight of approximately 80-82 kilodaltons (kDa). β_I was detected as a doublet in nearly all of the osteoblasts examined (Figure 1A). This doublet may represent different phosphorylation forms of the β_I isozyme, as reported for several of the PKC isozymes⁽³⁾.

The novel isozymes examined in osteoblasts were PKC- δ , ϵ , η , and θ (Figure 1B). Like the conventional α and β_1 isozymes, PKC- ϵ was expressed in each of the osteoblasts screened; the immunoreactivity of ϵ differed widely, however, among the different osteoblasts. The other novel isozymes showed more varied expression between cell lines. Utilizing the anti-PKC- δ antibody raised against a peptide derived from the internal portion of PKC- δ , this isozyme was readily observable in the UMR-106 rat line, and minimally detectable in normal mouse osteoblasts and in the SaOS-2 and HOS-TE85 human lines (Figure 1B). Using the C-terminal PKC- δ antibody, δ was also readily detectable in the UMR-106 cells; among the other osteoblasts, δ was evident only in the normal osteoblasts (data not shown). PKC- η was expressed in the normal mouse osteoblasts, the UMR-106, SaOS-2 and HOS-TE85 cells and faintly in the ROS 24/1 and MG-63 lines; it was not found in the ROS 17/2.8 cells. PKC- θ was expressed only in the SaOS-2 and HOS-TE85 lines; on some blots, θ was also detectable in the ROS 24/1 line (data not shown).

As was found for the conventional isozymes, the molecular weights of the novel PKC isozymes detected in osteoblasts were consistent with those reported for these isozymes in other tissues^(3,4-6,29). PKC- δ migrated with a molecular weight of approximately 76 kDa. PKC- ϵ ran at approximately 90 kDa and like β_1 , was detected as a doublet in some of the osteoblasts examined (Figure 1B). PKC- η appeared as a doublet in the human cell lines in which it was expressed. The lower band of these doublets migrated to the same extent as did the single band observed in the other η -expressing osteoblasts (Figure 1B). The lower band corresponded to 75 kDa and the upper band to 81 kDa. PKC- θ had a molecular weight of approximately 79 kDa.

Expression of the atypical ζ and ι/λ isozymes was also examined in the osteoblasts. Because PKC- ζ and ι/λ share a common C-terminus^(7,8), the C-terminal PKC- ζ antibody cross-

reacts with PKC- ι/λ , and both isozymes are seen on the same blot. PKC- ζ was detected as a single band at approximately 75 kDa and PKC- ι/λ as a single band at approximately 65 kDa; these molecular weights agree with those reported for these isozymes in other tissues⁽⁷⁾. As shown in Figure 1C, both isozymes were expressed in each of the osteoblasts screened. The highest molecular weight band (C, 80 kDa) seen on this blot may represent a conventional isozyme, possibly PKC- α ; cross-reactivity of C-terminal ζ antibodies with conventional isozymes has been reported previously^(30,31). As was observed with the PKC- α -specific antibody (Figure 1A), this isozyme was expressed in each of the osteoblasts screened.

Sensitivity of PKC isozymes to prolonged phorbol ester treatment

Phorbol esters are widely employed as pharmacological tools to explore the role of PKC in cellular processes. To obtain interpretable information from the physiological results, however, one must have knowledge of which isozymes the phorbol ester acts on as well as the time course over which these effects are manifested. To characterize the action of phorbol esters in osteoblasts, the effect of 1-48 h phorbol treatment of the cells on PKC isozyme expression was examined. Normal mouse osteoblasts and the UMR-106 rat osteoblastic cells were selected for these time course studies. No measurable effect of phorbol ester treatment on cell growth was observed over the time course examined.

As shown in Figure 2, the time courses and the extent of the responses of the conventional α and β_I isozymes to 1 μ M PDB differed somewhat in the normal mouse osteoblasts and the UMR-106 osteoblastic cells; the sensitivity of the β_{II} isozyme was not examined due to the relatively low expression of this isozyme in osteoblasts. In the normal osteoblasts, slight down-

regulation of PKC- α was detectable after only a 1 h treatment with 1 μ M PDB (77% of 1 h control. Note: Time course calculations are based on time-matched controls). Down-regulation of PKC- β_1 was first evident at 3 h, having decreased to 64% of control. At 6 h, both PKC- α and - β_1 fell to approximately 48% of control levels. Down-regulation of both isozymes was maintained at approximately 50% at all later time points examined (Figure 2B).

In the UMR-106 line, down-regulation of PKC- α and - β_1 was first detectable following 3 h treatment with 1 μ M PDB (Figure 2); at 3 h, α and β_1 were decreased to 78 \pm 4% (mean \pm standard error) and 71 \pm 7% of control, respectively (Figure 2B). At 6 h, both isozymes were down-regulated to a slightly greater extent, falling to 63 \pm 4% (α) and 63 \pm 13% (β_1) of control levels. PKC- α continued to decline to 31 \pm 6% and 18 \pm 4% of control at 12 and 24 h, respectively, but then reached a plateau (17 \pm 8% of control) with 48 h PDB treatment. Levels of PKC- β_1 also continued to decline with longer treatment periods, dropping to 38 \pm 15%, 23 \pm 7%, and 16 \pm 4% of control at 12, 24, and 48 h, respectively.

The novel isozymes showed a range of sensitivities to 1 μ M PDB treatment, with the δ and ϵ isozymes being more completely down-regulated than PKC- η (Figure 3). In the normal osteoblasts, PKC- δ was dramatically down-regulated after 1 h PDB treatment (56% of control) and further down-regulated at 3 h (24% of control). In examining the Western blot, PKC- δ appears to be completely down-regulated at all subsequent time points. The densitometer, however, detects some residual immunoreactivity at these later time points. PKC- ϵ was also completely down-regulated by prolonged phorbol treatment in normal osteoblasts, although the time course was somewhat different than for δ . Down-regulation of PKC- ϵ was first apparent at 6

h and was complete at this time point. As for PKC- δ , down-regulation of PKC- ϵ was maintained at all subsequent time points.

Expression of the δ and ϵ isozymes in UMR-106 osteoblastic cells was affected in a parallel manner by prolonged PDB treatment (1 μ M) (Figure 3). PKC- δ was partially down-regulated after 1 h PDB treatment (68 \pm 3% of control), further down-regulated at 3 and 6 h (18 \pm 5% and 11 \pm 3% of control, respectively), and completely down-regulated at all subsequent time points (<5% of control). Down-regulation of PKC- ϵ was first detectable in the UMR-106 cells at 3 h (62 \pm 6% of control), and declined progressively with 6, 12, 24, and 48 h treatments (30 \pm 6, 15 \pm 4%, 12 \pm 2%, and 7 \pm 1% of control, respectively).

The novel η isozyme was unique in that it was affected by phorbol ester treatment in two distinct ways. These effects were seen in both the normal mouse osteoblasts and the UMR-106 cell line. First, phorbol treatment caused a slight decrease in the mobility of PKC- η (Figure 3A). This effect, seen as a slight upward shift in the PKC- η band, was apparent at all time points examined and may be due to phosphorylation of η with phorbol treatment. Second, phorbol treatment caused a down-regulation of the η isozyme (Figure 3A, B), as described above for the other novel isozymes. In normal osteoblasts, PKC- η was only moderately sensitive to prolonged PDB treatment. Down-regulation was apparent at 6 h (75% of control) but failed to progress with longer treatment periods. In contrast, in the UMR-106 line, down-regulation of η was first detectable at 6 h (84 \pm 27% of control) and declined further at later time points, falling to 43 \pm 12%, 38 \pm 12%, and 28 \pm 3% of control levels at 12, 24, and 48 h, respectively.

Unlike the conventional and novel PKC isozymes, the atypical ζ and ι/λ isozymes were insensitive to prolonged phorbol ester treatment at all time points examined (Figure 4A, B). This

insensitivity was observed in both the normal mouse osteoblasts and the UMR-106 cell line. As seen in Figure 4, the uppermost band observed in blots with the anti-PKC ζ antibody (C) was down-regulated by phorbol ester treatment. In the normal osteoblasts, down-regulation was detectable after 1 h PDB treatment (69% of control). Isozyme levels were slightly higher at 3 h but were still lower than in control cells (81% of control). At 6 h, levels fell to 38% of control, and remained at 45-60% of control at all subsequent time points. In the UMR-106 cells, down-regulation of this isozyme was apparent after 3 h PDB treatment (78 \pm 5% of control). Isozyme levels declined progressively at later time points falling to 65 \pm 10%, 40 \pm 6% and 26 \pm 7% of control at 6, 12, and 24 h, respectively. The down-regulation observed at 24 h was maintained with 48 h PDB treatment (24 \pm 6% of control). These results are consistent with those obtained using the PKC- α -specific antibody (Figure 2).

The time course studies described above were carried out with a single dose (1 μ M) of the phorbol ester PDB. This suggested that the more moderate down-regulation observed for the conventional as compared to the novel isozymes could be a dose-related effect. Moreover, it was possible that the lack of down-regulation of the atypical ζ and ι/λ isozymes was dose related. To address this issue, the sensitivity of the isozymes to 24 or 48 h treatment with two additional doses of PDB was examined. The conventional (α , β_1) and novel (ϵ , η) isozymes showed a greater degree of down-regulation following 48 h treatment with 1 μ M versus 0.3 μ M PDB; PKC- δ and η were maximally down-regulated even with 0.3 μ M PDB (Figure 5). Following treatment with 3 μ M PDB, the conventional and novel isozymes were down-regulated to approximately the same extent as with 1 μ M PDB treatment. As was observed with 1 μ M PDB, 48 h treatment with 0.3 μ M or 3 μ M PDB failed to down-regulate the atypical PKC isozymes. Similar dose-response

results were obtained following 24 h treatment with 0.3 μM , 1 μM , or 3 μM PDB (data not shown).

To examine the specificity of the phorbol ester-mediated down-regulation, experiments were carried out with the inactive phorbol ester 4 α -phorbol 12,13-didecanoate (4 α -PDD). 48 h treatment of UMR-106 cells with 1 μM 4 α -PDD had no effect on expression of the conventional, novel, or atypical isozymes as compared to vehicle-treated cells (data not shown).

Phorbol ester-mediated down-regulation of PKC is associated with an increased rate of degradation of PKC⁽¹⁴⁾. This down-regulation has been reported to be reversible in other cell types⁽³²⁾. To examine the reversibility of the PDB-mediated down-regulation in osteoblasts, UMR-106 cells were exposed to vehicle for 48 h, 1 μM PDB for 48 h, or 1 μM PDB for the first 24 h and vehicle for the final 24 h. The phorbol-sensitive conventional (α , β_1) and novel (δ , ϵ , η) isozymes were each significantly down-regulated by 48 h PDB treatment (Figure 6), as was seen in the time course and dose response studies. When the PDB was removed at 24 h, however, all isozymes, except PKC- η , had recovered some extent by 48 h. PKC- α was restored nearly to control levels, while PKC- β_1 , - δ , and - ϵ recovered to 40-70% of control. The phorbol-sensitive isozyme detected with the anti- ζ antibody (C) recovered to 63% of control.

DISCUSSION

The current results indicate that there is a characteristic pattern of PKC isozyme expression in osteoblasts across species and in both normal and osteosarcoma-derived cells. The current studies were carried out with whole cell lysates containing both cytosolic and particulate fractions. In preliminary studies⁽³³⁾, we have found differences in the subcellular distribution of several isozymes in both normal mouse osteoblasts and UMR-106 cells (under resting conditions). The PKC isozymes found to be present in all of the osteoblasts screened are PKC- α , - β_I , - ϵ , - ζ and - ι/λ (Figure 1). PKC- β_{II} is expressed in each of the osteoblasts except the ROS 24/1 rat line, which is less osteoblastic than the other cell lines examined^(26,34). The novel δ , η , and θ isozymes are detectable only in some of the osteoblasts examined. The variability in expression of these isozymes may be a reflection of the different stages of differentiation of the osteoblasts screened⁽³⁴⁻³⁹⁾. Conversely, the variable expression of PKC- δ , - ϵ and - η may be an indication that these isozymes are not important for the uniquely osteoblastic functions of these cells.

Few studies of PKC isozyme expression in bone have been conducted. Sakai *et al.*⁽⁴⁰⁾ examined conventional PKC isozyme expression in MC3T3-E1 mouse osteoblastic cells. MC3T3-E1 cells, like the osteoblastic cells examined in our study, express PKC- α and - β , but not γ (the β antibody used detected both β_I and β_{II}). Isozyme expression in osteoclast-like cells has also been partially characterized. Teti *et al.*⁽⁴¹⁾ examined isozyme expression in both human osteoclast-like cells (isolated from giant cell tumors of bone) and freshly isolated rat osteoclasts. Both types of osteoclast-like cells express PKC- α , - δ , and - ϵ , but not β . (As in the Sakai *et al.* study, the anti-PKC- β antibody was capable of detecting both β_I and β_{II}). Expression of the other known PKC isozymes was not determined.

The study presented here is the first thorough characterization of the isozymes present in osteoblastic cells and indicates the difference between bone and other tissues, such as brain, which expresses all the known isozymes of PKC⁽¹⁻⁸⁾ and kidney which expresses PKC- α , - δ , - ϵ , and - ζ ⁽³⁾. The α , β_I/β_{II} , δ , ϵ , and ζ isozymes are ubiquitously expressed^(2,3), and of these, all but PKC- δ are commonly expressed in osteoblasts. None of the osteoblasts screened express PKC- γ . This result is not surprising, however, as this isozyme has been detected primarily in the central nervous system^(1,2). A broad determination of the tissue expression of the other isozymes examined in this study, PKC- η , - θ , and - ι/λ has not yet been carried out. Initial studies suggest that PKC- η and - θ have a rather limited distribution, with η being expressed predominantly in the skin and lung^(4,5) and θ largely in skeletal muscle⁽⁶⁾. Both isozymes are expressed at a lower extent in the brain and spleen⁽⁴⁻⁶⁾. In contrast to η and θ , initial studies of PKC ι/λ expression suggest that this isozyme is widely expressed, but most notably in the lung, brain, and kidney^(7,8).

As expected, there are differences in phorbol ester sensitivity among the different osteoblast PKC isozymes (Figures 2-4). The conventional and novel isozymes are down-regulated by prolonged phorbol ester treatment, but the atypical isozymes are not. These results are consistent with the ability of these classes to bind phorbol esters. The conventional and novel isozymes possess the two cysteine-rich zinc finger motifs in the conserved C1 PKC domain necessary to bind DAG and phorbol esters⁽¹⁻³⁾; atypical isozymes lack one of the two zinc fingers, however, and are unable to bind DAG or phorbol esters^(7,8,10-13).

No major differences in phorbol sensitivity are apparent in comparing the results obtained in the normal osteoblasts and the UMR-106 cell line (Figures 2-4). All of the isozymes that undergo phorbol-induced down-regulation in normal cells also do so in the UMR-106 cell line.

There are differences as to the extent of down-regulation observed in the two cell types, however; for example, η appears to be down-regulated to a greater extent in the UMR-106 cells than in the normal osteoblasts. PKC- η exhibits both a change in mobility and subsequent down-regulation in both cell types, though, indicating that the general response to prolonged phorbol ester treatment is the same in these two osteoblast models.

Down-regulation occurs quite rapidly for nearly all of the phorbol-sensitive osteoblast isozyms (Figures 2-4). All sensitive isozyms, with the exception of PKC- η in normal osteoblasts, are significantly diminished following 6 h phorbol treatment. This rapid time course of down-regulation has implications for experiments in which a phorbol ester is used as a tool to presumably activate PKC. Unless the exposure times are very short, the results may actually be due to a down-regulation rather than an activation, leading to an opposite interpretation for the role of PKC in the process. Another possibility in such experiments is that the initial activation, rather than the subsequent down-regulation of the kinase activity, is the critical event.

Although both the conventional and novel isozyms are down-regulated by prolonged phorbol treatment in osteoblasts, the extent of the down-regulation observed for these two classes is somewhat different (Figures 2,3). The conventional α and β_1 isozyms are less sensitive to prolonged phorbol ester treatment than the novel isozyms, particularly PKC- δ and - ϵ . This difference in sensitivity does not appear to be a dose-related effect as 24 or 48 h treatment with 3 μ M PDB produces approximately the same degree of down-regulation as 1 μ M PDB (Figure 5). Treatment with a lower dose of PDB, however, is less effective in down-regulating some isozyms (Figure 5), indicating that phorbol-induced down-regulation is a concentration-dependent effect in osteoblasts. Another possibility is that the conventional isozyms might be further down-regulated

with longer phorbol treatments (>48 h); this seems unlikely, though, because PKC- α and β_1 isozyme levels appear to plateau with 24 and 48 h phorbol treatments (Figure 2). Differences in the extent of phorbol-induced down-regulation of conventional and novel isozymes have been reported previously⁽⁴²⁾, but the reason for this difference is not clear.

The phorbol ester-induced down-regulation of PKC isozymes observed in osteoblasts is a specific and reversible effect. The inactive phorbol 4 α -PDD fails to down-regulate PDB-sensitive isozymes (data not shown). In addition, the atypical isozymes are insensitive to the phorbol treatments that significantly down-regulate the conventional and novel isozymes (Figure 4). The reversibility of the phorbol-induced down-regulation observed in osteoblasts is consistent with the means by which this down-regulation is effected. Prolonged phorbol treatment increases the net rate of degradation of PKC⁽¹⁴⁾. When phorbol is removed from the cellular environment, however, degradation declines to its basal rate and due to the ongoing synthesis of PKC, isozyme levels are eventually restored. The precise molecular mechanism responsible for the phorbol-induced down-regulation of PKC is not known.

As mentioned above, the failure of prolonged phorbol treatment to down-regulate the atypical ζ and ι/λ isozymes in osteoblasts is consistent with the inability of these isozymes to bind phorbol ester^(8,10-13). A similar insensitivity of PKC- ζ to phorbol has been described in other cell types, including renal mesangial cells⁽³²⁾, melanocytes^(42,43), and Jurkat T lymphoma cells⁽⁴⁴⁾. Phorbol-induced down-regulation of ζ has also been reported⁽⁴⁵⁻⁴⁷⁾, but in many of these studies, the authors employed a C-terminal PKC- ζ antibody, as used in our studies. For example, in a recent study carried out in urinary tract smooth muscle cells⁽⁴⁷⁾, the cross-reactive anti-PKC- ζ antibody was utilized to examine the sensitivity of the ζ isozyme to 24 h phorbol myristate acetate

(PMA) treatment. As in our studies, the Western blot revealed three bands at approximately 80, 75, and 65 kDa. The 80 kDa ζ -reactive band was down-regulated by 24 h PMA treatment, but the other two bands were unaffected by this treatment. The 80 kDa band was considered to be PKC- ζ , and no mention was made of the other two bands observed on the blot. Therefore, when results obtained with C-terminal PKC- ζ antibodies are interpreted, the ability of this antibody to cross-react with phorbol-sensitive conventional isozymes must be considered.

The studies carried out thus far demonstrate that osteoblasts have a characteristic PKC isozyme profile, including both phorbol ester-sensitive and -insensitive isozymes. It will be of interest to now determine the functional roles of the specific isozymes in osteoblastic functions. In preliminary studies, we have found that phorbol ester treatments that result in down-regulation of PKC isozymes markedly diminish PTH-stimulated interleukin-6 secretion⁽⁴⁸⁾. Based on the studies described here, it is now reasonable to determine which particular isozyme(s) mediates this effect.

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Figure 1. PKC isozyme expression in osteoblasts. Whole cell lysates from normal mouse osteoblasts (Normal OB) and rat and human osteoblastic osteosarcoma cells were subjected to Western blot analysis (50 $\mu\text{g}/\text{lane}$) with isozyme-specific anti-PKC antibodies. Blots utilizing antibodies to the **A.** conventional (α , β_{I} , β_{II} , γ), **B.** novel (δ , ϵ , η , θ), and **C.** atypical (ζ , ι/λ) isozymes are shown. (PKC- ι/λ is referred to simply as PKC- ι in this and subsequent figures). A homogenate prepared from a whole mouse brain (MBrStd) was included as a positive control.

Figure 2. Time course of phorbol ester-induced down-regulation of conventional PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle or 1 μM PDB. Whole cell lysates were subjected to Western blot analysis (normal osteoblasts: 50 $\mu\text{g}/\text{lane}$; UMR-106 cells: 25 $\mu\text{g}/\text{lane}$ (α), 75 $\mu\text{g}/\text{lane}$ (β_{I})). **A.** Blots with anti-PKC- α and - β_{I} antibodies. Untreated control (Untrt Con); Vehicle-treated control (C); Phorbol treated (P). **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blots shown. The plot for the UMR-106 cells is the mean \pm standard error (SE) of 3 experiments; a representative blot for each isozyme is shown.

Figure 3. Time course of phorbol ester-induced down-regulation of novel PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle or 1 μM PDB. Whole cell lysates were subjected to Western blot analysis (normal osteoblasts: 50 $\mu\text{g}/\text{lane}$; UMR-106 cells: 25 $\mu\text{g}/\text{lane}$ (δ , ϵ), 75 $\mu\text{g}/\text{lane}$ (η)). **A.** Blots with anti-PKC- δ , - ϵ and - η antibodies. Untreated control (Untrt Con); Vehicle-treated control (C); Phorbol

treated (P). **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blots shown. The plot for the UMR-106 cells is the mean \pm SE of 3 experiments; a representative blot for each isozyme is shown.

Figure 4. Phorbol ester treatment fails to down-regulate atypical PKC isozymes in osteoblasts. Normal mouse osteoblasts or UMR-106 cells were treated for 1, 3, 6, 12, 24, or 48 h with vehicle or 1 μ M PDB. Whole cell lysates were subjected to Western blot analysis (50 μ g/lane). **A.** Blots with the anti-PKC- ζ antibody, which cross-reacts with PKC- ι and a conventional isozyme (C). Untreated control (Untrt Con); Vehicle-treated control (C); Phorbol treated (P). **B.** Densitometric analysis of blots. Values are plotted as a percent of the appropriate time-matched control. The plot for normal osteoblasts was derived from the blot shown. The plot for the UMR-106 cells is the mean \pm SE of 3 experiments; a representative blot is shown.

Figure 5. Dose response for PDB-induced down-regulation of PKC isozymes in osteoblasts. UMR-106 cells were treated with vehicle or 0.3 μ M, 1 μ M, or 3 μ M PDB for 48 h. Whole cell lysates were subjected to Western blot analysis (25 μ g/lane (α , δ , ϵ); 50 μ g/lane (ζ - ι); 75 μ g/lane (β _I, η) with isozyme-specific anti-PKC antibodies. **A.** Blots for conventional, novel, and atypical isozymes. **B.** Densitometric analysis of blots shown in **A.**

Figure 6. Phorbol ester-sensitive PKC isozymes recover following withdrawal of the phorbol ester. UMR-106 cells were treated for 48 h, with a medium change at 24 h. The cells were treated

with vehicle for 48 h (V-V), 1 μ M PDB for 48 h (P-P), or 1 μ M PDB for the initial 24 h period and vehicle for the final 24 h (P-V). Whole cell lysates were subjected to Western blot analysis (25 μ g/lane (α , δ , ϵ); 50 μ g/lane (ζ - ι); 75 μ g/lane (β _I, η)) with isozyme-specific anti-PKC antibodies. **A.** Blots for conventional, novel, and atypical isozymes. **B.** Densitometric analysis of blots shown in **A.**

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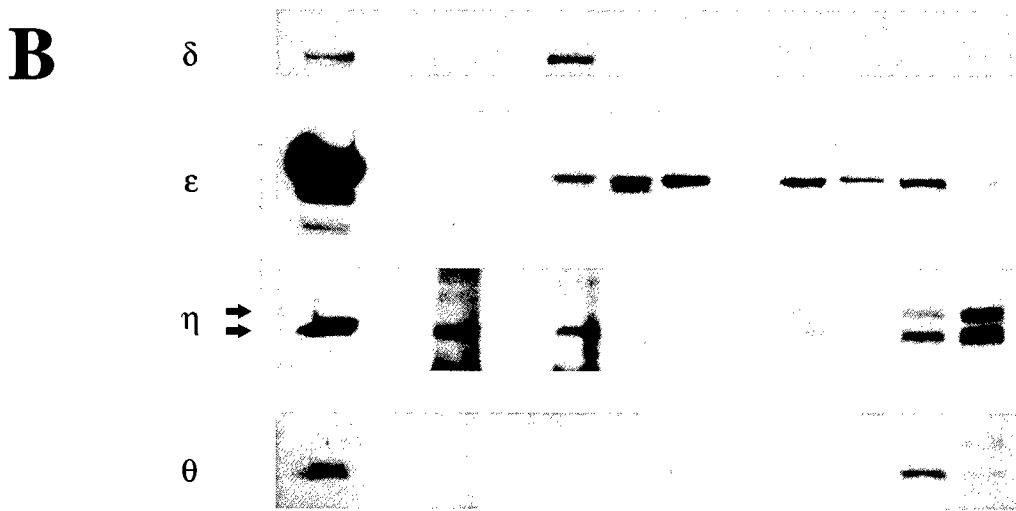
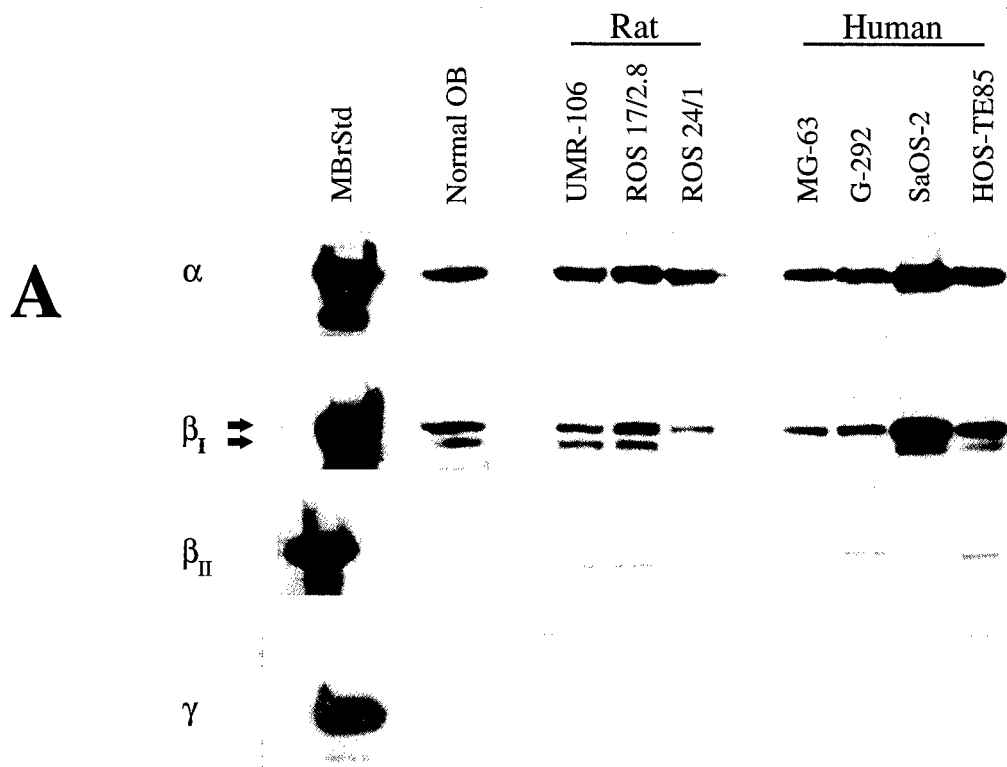
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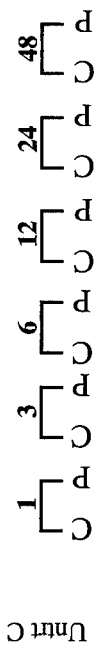
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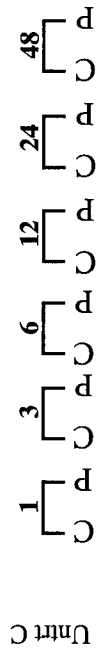


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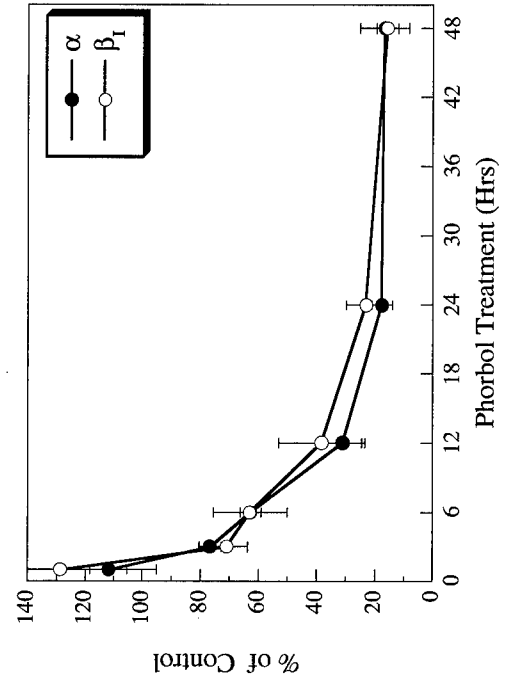
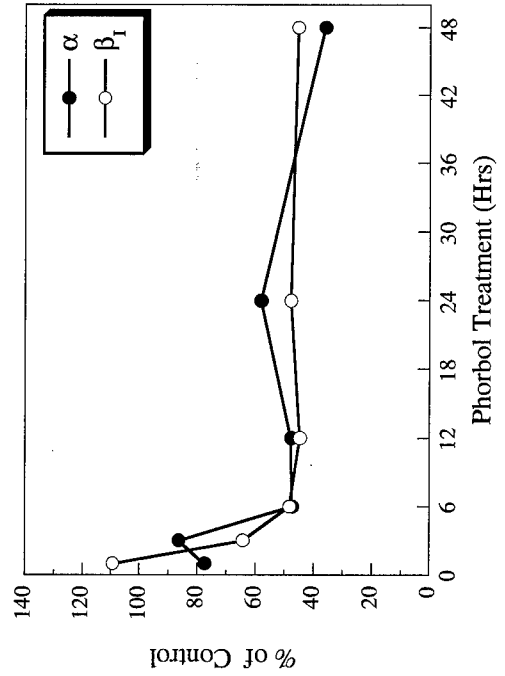
Normal Mouse Osteoblasts



UMR-106 Osteoblastic Cell Line

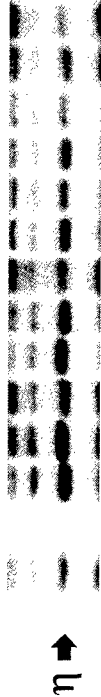
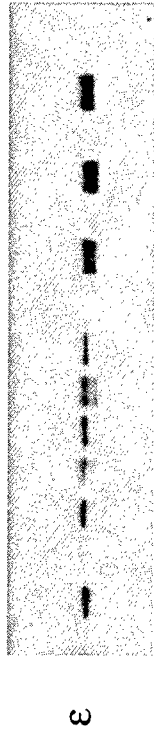
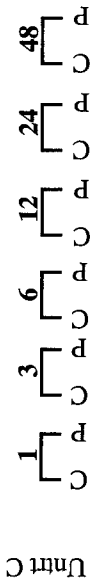


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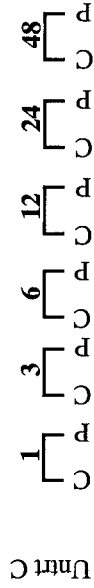


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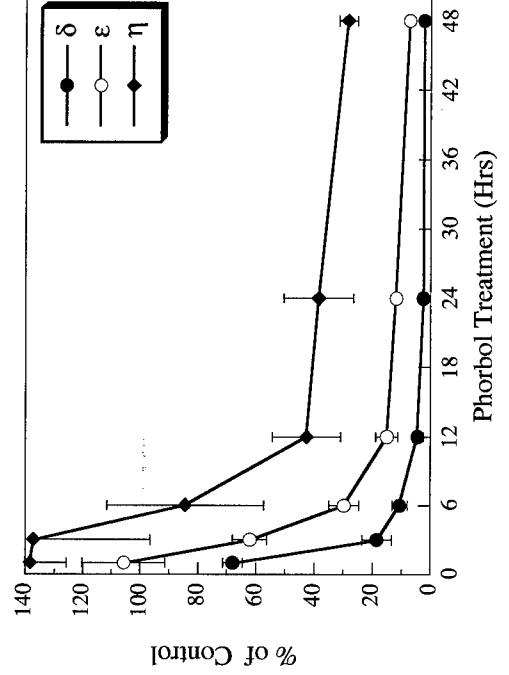
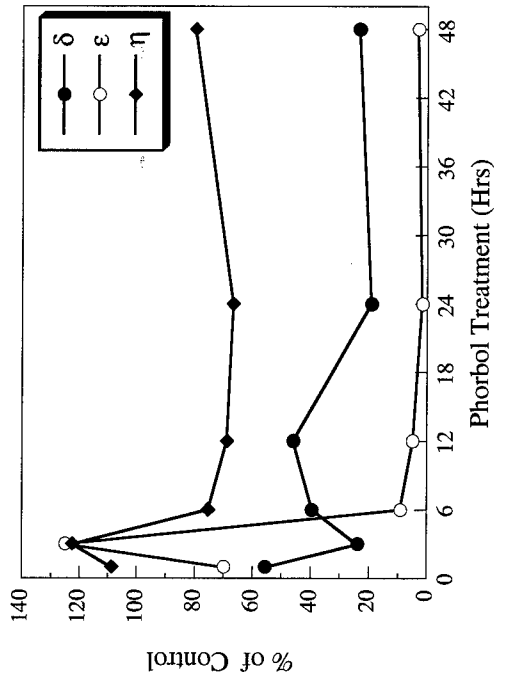
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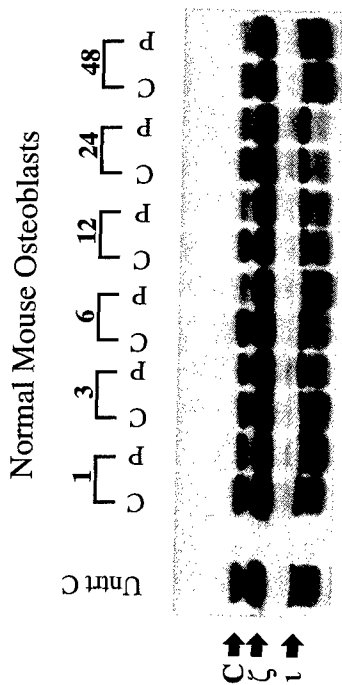
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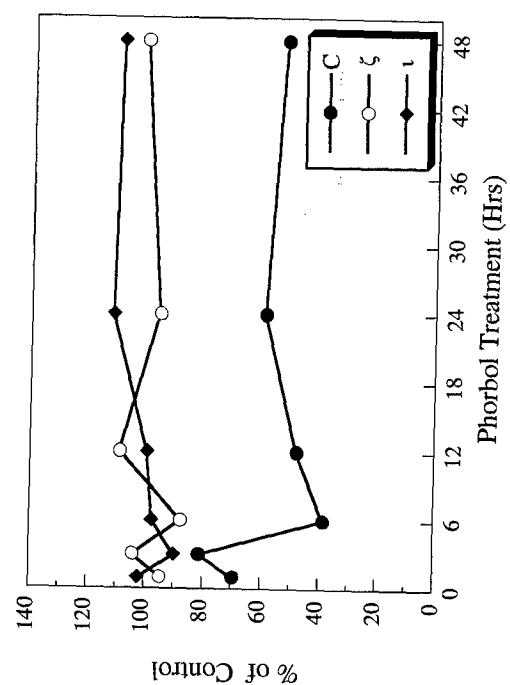
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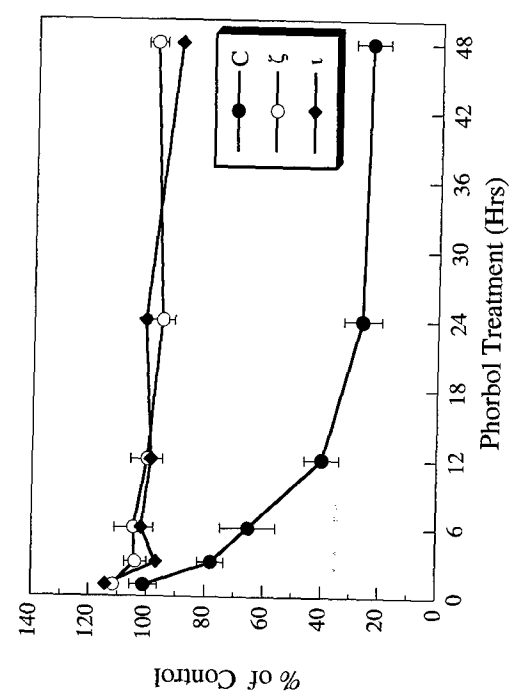
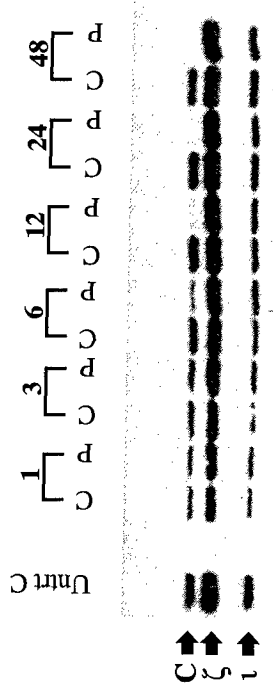
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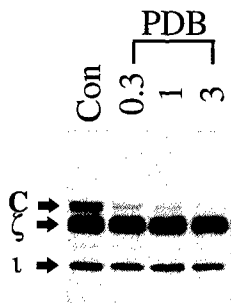
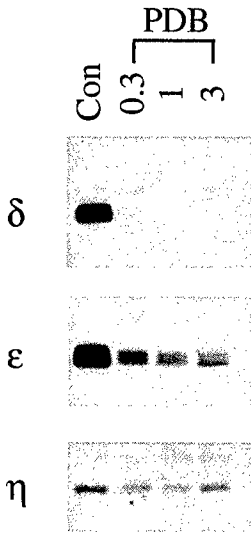
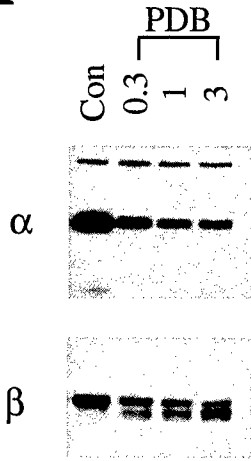
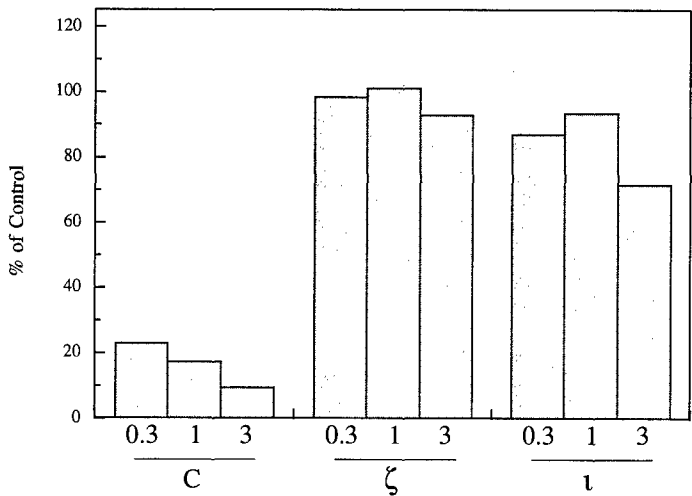
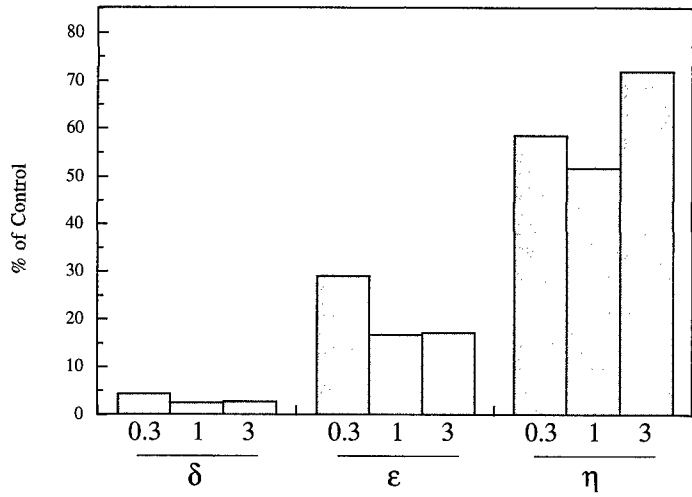
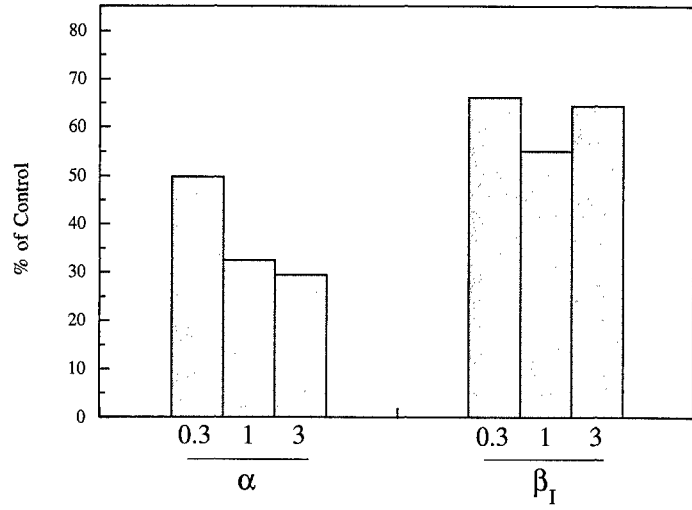


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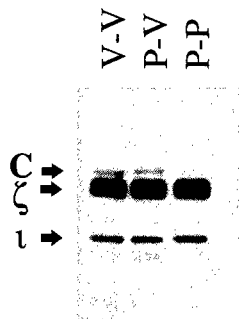
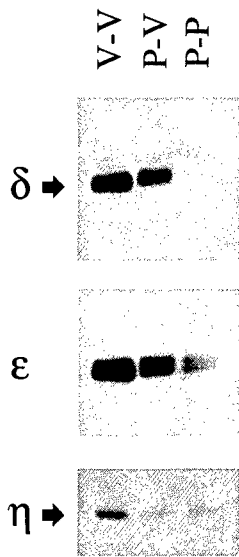
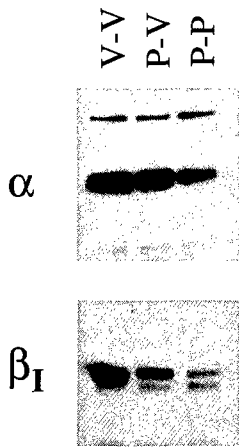


UMR-106 Osteoblastic Cell Line

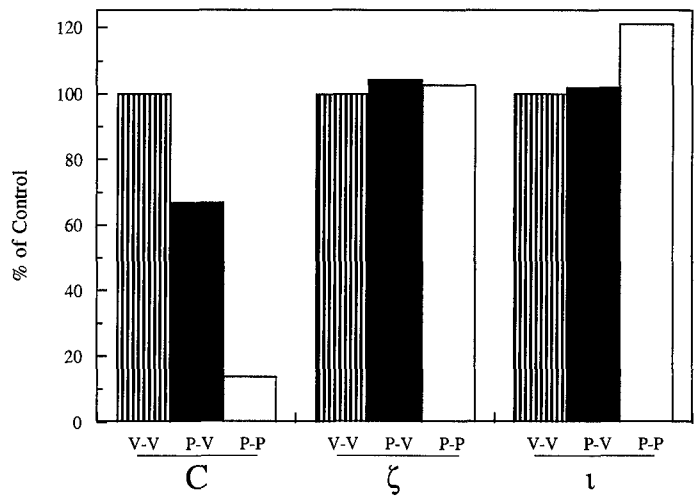
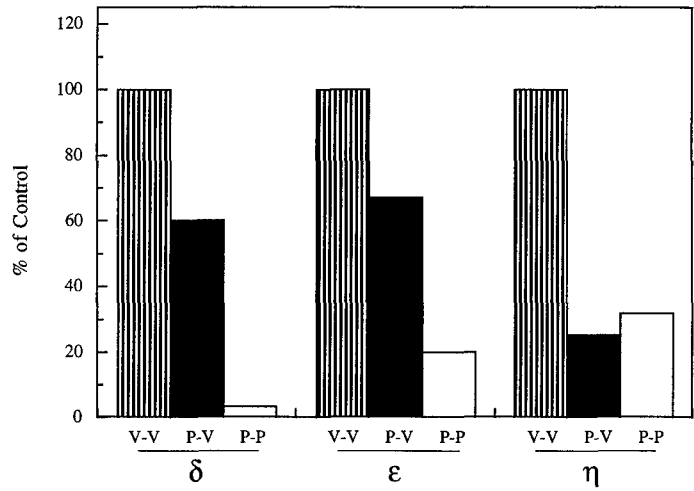
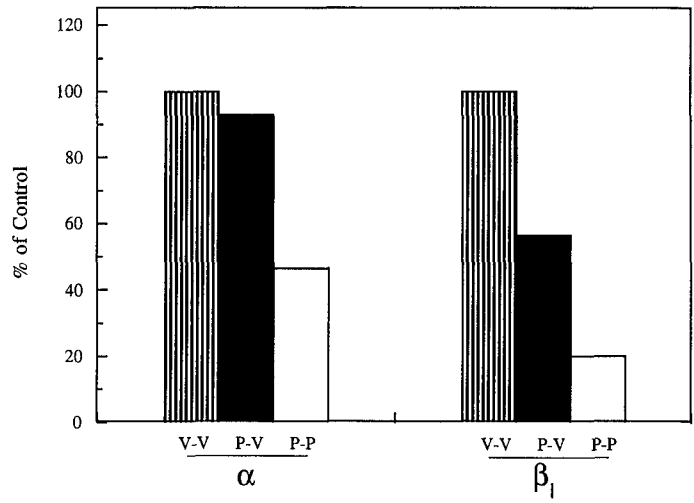


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BIBLIOGRAPHY

Please note: Jennifer L. Sanders was the only person to receive pay (stipend) from the negotiated effort.

Full Papers:

1. Sanders JL, Stern PH 1996 Expression and phorbol ester-induced down-regulation of protein kinase C isozymes in osteoblasts. *J Bone Miner Res*, accepted for publication.

Abstracts:

1. Lee SK, Sanders JL, Hunzicker-Dunn M, Ohno S, Stern PH 1994 Differential effects of phorbol ester and parathyroid hormone on protein kinase C isozymes in UMR-106 and primary osteoblastic cells. *J Bone Miner Res* 9(Suppl 1):S238.
(Poster presentation at the 1994 Annual Meeting of the American Society for Bone and Mineral Research, Kansas City, MO; also at the 1995 Chicago Signal Transduction Symposium, Chicago, IL, and the 1995 Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics (Great Lakes Chapter), Chicago, IL).
2. Sanders JL, Tarjan G, Strieleman PJ, Stern PH 1995 Protein kinase C mediates interleukin-6 production by parathyroid hormone in UMR-106 cells. *J Bone Miner Res* 10(Suppl 1):S385.
(Poster presentation at the 1995 Annual Meeting of the American Society for Bone and Mineral Research, Baltimore, MD).
3. Sanders JL, Stern PH 1996 Expression and phorbol-ester sensitivity of protein kinase C isozymes in osteoblasts. *FASEB J* 10:A680.
(Poster presentation at Experimental Biology 1996, Washington, D.C.; also at the 1996 Chicago Signal Transduction Symposium, Chicago, IL, and the 1996 Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics (Great Lakes Chapter), Chicago, IL).
4. Sanders JL, Stern PH 1996 Protein kinase C isozymes in osteoblasts: expression and phorbol ester-induced down-regulation. *J Bone Miner Res*.
(To be presented as a poster at the 1996 Annual Meeting of the American Society for Bone and Mineral Research, Seattle, WA).



DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 Apr 97


MEMORANDUM FOR Administrator, Defense Technical Information
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant Number DAMD17-94-J-4145. Request the limited distribution statement for Accession Document Number ADB215556 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322.

FOR THE COMMANDER:


GARY R. GILBERT
Colonel, MS
Deputy Chief of Staff for
Information Management

Completed 170-00