Antisense Oligonucleotides Targeted against Protein Kinase Cβ and CβII Block 1,25-(OH)2D3-induced Differentiation*

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Robert U. Simpson‡‡, Timothy D. O’Connell‡, Quintin Pan‡, Judy Newhouse‡, and Martha J. Somerman‡‡‡

From the ‡Department of Pharmacology and ‡‡Department of Periodontics/Prevention/Geriatrics, the University of Michigan, Ann Arbor, Michigan 48109-0632

It is now recognized that protein kinase C (PKC) plays a critical role in 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) promotion of HL-60 cell differentiation. In this study, the effects of phosphorothioate antisense oligonucleotides directed against PKCα, PKCβ, PKCβII, and PKCβIII on HL-60 promyelocyte cell differentiation and proliferation were examined. Cellular differentiation was determined by nonspecific esterase activity, nitro blue tetrazolium reduction, and CD14 surface antigen expression. Differentiation promoted by 1,25-(OH)2D3 (20 nM for 48 h) was inhibited similarly in cells treated with PKCβ antisense (30 μM) 24 h prior to or at the same time as hormone treatment (86 ± 9% inhibition; n = 4 versus 82 ± 8% inhibition; n = 4 (mean ± S.E.), respectively). In contrast, cells treated with PKCβ antisense 24 h after 1,25-(OH)2D3 were unaffected and fully differentiated. PKCα antisense did not block 1,25-(OH)2D3 promotion of HL-60 cell differentiation. Next, the ability of PKCβI and PKCβIII-specific antisense oligonucleotides to block 1,25-(OH)2D3 promotion of cell differentiation was examined. PKCβII antisense (30 μM) completely blocked CD14 expression induced by 1,25-(OH)2D3, whereas PKCβII antisense had little effect. Interestingly, PKCβII antisense blocked differentiation by 87 ± 7% (n = 2, mean ± S.D.) but had no effect on 1,25-(OH)2D3 inhibition of cellular proliferation. These results indicate that the effects of 1,25-(OH)2D3 on HL-60 cell differentiation and proliferation can be dissociated by blocking PKCβII expression.

The hormone 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) regulates the growth and maturation of numerous organs and cell types. 1,25-(OH)2D3 is involved in the control of calcium and phosphorus homeostasis, muscle function, immunity, endocrine secretions, and neurotransmission (1). It is accepted that, in part, this hormone alters cell function by enhancing or repressing expression of specific genes (2, 3). Other studies have revealed that 1,25-(OH)2D3 regulates cellular processes without altering gene expression (4). These observations suggest that nongenomic effects of the hormone occur and result in the rapid alteration of cell membrane phospholipid metabolism and intracellular calcium concentrations (5, 6). Although the exact mechanism by which 1,25-(OH)2D3 promotes HL-60 cell differentiation is not fully understood, a number of studies from our laboratory and others have implicated protein kinase C (PKC) as a critical component of this process (7–9).

PKC is a family of serine-threonine protein kinases, which play major roles in regulation of many cellular processes. To date, 11 PKC isoenzymes have been characterized and classified into three groups based on their structure and activation requirements (10, 11). The classical PKCs, PKCα, PKCβ, PKCβII, and PKCγ require calcium for activation. A second class of PKCs has been termed the novel PKCs and consist of PKCδ, PKCε, PKCη, and PKCζ (11). These novel PKCs do not have a calcium binding motif, and therefore calcium is not required for activation. The third class of PKCs are called the atypical PKCs and include PKCδ, PKCμ, and PKCζ. These PKCs differ significantly in structure to the other PKCs. Furthermore, atypical PKCs do not respond to phorbol ester activation.

The importance of PKC in 1,25-(OH)2D3 promotion of HL-60 cells along the monocyte/macrophage pathway is now appreciated. Our laboratory reported that 1,25-(OH)2D3 increases PKC levels in HL-60 cells (7). Additionally, we found that classical inhibitors of PKC, H-7 and staturosporine, block the ability of 1,25-(OH)2D3 to promote HL-60 cell differentiation (12, 13). Using similar PKC inhibitors, PKC activation by 1,25-(OH)2D3 has been shown to be involved in skin, heart, skeletal muscle, and renal cell gene expression and function (14–17). Unfortunately, such chemical inhibitors are of little use in determining isoenzyme specificity for a cellular transduction mechanism. Recent studies have used overexpression and antisense techniques to provide evidence that PKCβ is, to some extent, the isoenzyme involved in 1,25-(OH)2D3 promotion of HL-60 cell differentiation (18, 19). In this study, we showed that increased PKCβII levels by 1,25-(OH)2D3 is required to promote HL-60 cell differentiation. Interestingly, PKCβII antisense had no effect on 1,25-(OH)2D3 inhibition of HL-60 cell proliferation. Our report shows that increases in PKCβII levels and activation are important events in 1,25-(OH)2D3 promotion of cell differentiation. Moreover, we suggest that the events leading to cellular differentiation most likely require protein phosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals—1,25-(OH)2D3 was purchased from Tetrionics Inc. (Madison, WI). Vitamin D3 metabolite purity and structural integrity were confirmed by high performance liquid chromatography and UV spectroscopy. All other reagents were reagent grade or better.

Cell Culture—HL-60 promyelocytic leukemia cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% horse serum, 1000
levels as compared with cells exposed to 1,25-(OH)₂D₃ alone. Importantly, cells treated with PKCβ antisense (lane 4) exhibited a mark inhibition in 1,25-(OH)₂D₃ induction of PKCβ levels. PKCβ levels were decreased by 81 ± 9% (mean ± S.E.) relative to sense or oligonucleotide free cultures. The antibody used to detect PKCβ in this experiment was not specific for the splice isoenzymes βI or βII. As seen in Fig. 1, untreated (control) cells routinely exhibited minimal levels of PKCβ. Furthermore, PKCβ levels remain unchanged in uninduced (control) cells even after 48 h of PKCβ antisense treatment (n = 13, lanes 1 and 2, Fig. 1). This observation is expected, because PKCβ has a half-life of greater than 70 h. Therefore, blocking translation with PKCβ antisense would not greatly influence existing levels of PKCβ. As shown in Fig. 2, PKCβ levels were increased within 24 h of 1,25-(OH)₂D₃ treatment. Furthermore, PKCβ antisense significantly blocked 1,25-(OH)₂D₃ induction of PKCβ levels at 24 and 48 h of hormone treatment. Therefore, these results demonstrate that the PKCβ antisense oligonucleotide is able to block the induction of PKCβ levels by 1,25-(OH)₂D₃.

Specificity of PKCβ Antisense to Inhibit 1,25-(OH)₂D₃ Induction of PKCβ Protein Levels—HL-60 cells were treated with 20 nM 1,25-(OH)₂D₃ alone (C) or with PKCα sense, PKCα antisense, PKCβ sense, or PKCβ antisense (30 μM amount of either oligonucleotide) for 48 h. PKCβ levels were determined by Western blot analysis (Fig. 3A). PKCβ levels in cells treated with 1,25-(OH)₂D₃ and PKCα sense, PKCα antisense, or PKCβ sense were not significantly different from cells treated with 1,25-(OH)₂D₃ alone. As expected, PKCβ antisense was able to inhibit the induction of PKCβ by 1,25-(OH)₂D₃ (lane 5). Moreover, as shown in Fig. 3B, PKCα antisense was able to specifically block 1,25-(OH)₂D₃ enhancement of PKCα levels. Importantly, PKCβ antisense had no effect on 1,25-(OH)₂D₃ induction of PKCα levels. These results demonstrate that PKCβ antisense has specificity in blocking 1,25-(OH)₂D₃-induced increases of PKCβ. Western blot analysis of PKCβ and PKCβ/II splice isoenzymes was performed using specific antibodies. PKCβ/II protein levels were detectable using these specific antibodies, whereas PKCβ/II levels were not detectable. This observation in HL-60 cells is similar to ones reported previously (20, 21). Also, the PKCα antisense oligonucleotide at concentrations up to 60 μM had no effect on 1,25-(OH)₂D₃ promotion of cell differentiation (data not shown).

Dose Response of PKCβ Antisense to Inhibit 1,25-(OH)₂D₃ Induction of PKCβ Levels and Cell Differentiation—HL-60 cells were treated with 20 nM 1,25-(OH)₂D₃, and either 30 μM PKCβ sense or 1, 10, or 30 μM PKCβ antisense for 48 h. PKCβ levels were determined by Western blot analysis (Fig. 4A). As shown in Fig. 4A, a dose-dependent decrease in PKCβ levels was observed with increasing concentrations of PKCβ antisense. In this experiment an 85% decrease, as determined by scanning.
The effects of antisense constructs on HL-60 cell differentiation and the importance of the time of PKC\(\beta\) antisense addition, relative to 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) treatment, were also examined. HL-60 cells were treated with PKC\(\alpha\) sense or PKC\(\beta\) antisense 24 h prior to (open symbols) or at the same time (closed symbols) as 20 nM 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\). Cell differentiation was determined by nitro blue tetrazolium dye reduction (circles) and nonspecific esterase activity (squares) (Fig. 4B). HL-60 cells treated with 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) in the absence of oligonucleotide treatment were induced to differentiate to the same extent as cells pretreated or co-treated with PKC\(\beta\) sense (data not shown). Differentiation promoted by 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) was inhibited by 86 ± 9% in cells pretreated with PKC\(\beta\) antisense (30 \(\mu\)M) and 82 ± 8% in cells co-treated with PKC\(\beta\) antisense (Fig. 4B). Therefore, it is likely that the action of the antisense construct is not to lower existing PKC\(\beta\) levels but to block 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\)-induced increases in PKC\(\beta\) synthesis. However, if cells were first treated with 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) for 24 h prior to PKC\(\beta\) antisense, antisense treatment was ineffective in blocking 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) promotion of cell differentiation (hatched circles and squares; Fig. 4B). This observation suggests that 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) has induced sufficient de novo synthesis of PKC\(\beta\) within 24 h to render the antisense PKC\(\beta\) construct impotent. Thus, these experiments reveal that a relevant and required action of 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) in promoting HL-60 cell differentiation is to up-regulate PKC\(\beta\) levels by increasing the synthesis of the enzyme.

Effects of PKC\(\beta\)I and PKC\(\beta\)II Antisense on HL-60 Cell Differentiation—HL-60 cells were treated with vehicle, 20 nM 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\), or 20 nM 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) and PKC\(\beta\) sense, PKC\(\beta\) antisense, PKC\(\beta\)I sense, PKC\(\beta\)II antisense, or PKC\(\beta\)II sense (30 \(\mu\)M) for 72 h, and cell differentiation was determined by CD14 surface antigen expression using flow cytometry (Fig. 5). CD14 is a cell surface marker of mature monocytes/macrophages. Treatment with 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) significantly increased cell differentiation as shown by the substantial increase in CD14 expression (Fig. 5). PKC\(\beta\) sense did not affect 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\)-induced expression of CD14, whereas PKC\(\beta\) antisense completely blocked the ability of 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) to increase CD14 expression. This is consistent with all previous observations and again demonstrates that PKC\(\beta\) is essential for 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\)-induced differentiation of HL-60 cells. In Fig. 5, C and D, the potency of antisense constructs designed to hybridize specifically with PKC\(\beta\)I and PKC\(\beta\)II was examined. A complete block of 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\)-induced CD14 expression was observed with the PKC\(\beta\)II-specific antisense oligonucleotide (Fig. 5C). In contrast, PKC\(\beta\)II-specific antisense failed to reverse the enhanced expression of CD14 by 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) (Fig. 5D).

Effects of PKC\(\beta\)II Antisense on Cell Proliferation—Interestingly, PKC\(\beta\)II antisense did not block 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) inhibition of HL-60 cell proliferation (Fig. 6). Thus, these data show that blocking 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\)-stimulated increase in PKC\(\beta\)II decreased the induction of cell differentiation by 80% but had no effect on 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) inhibition of cell proliferation. Similar results were obtained with the less specific PKC\(\beta\) antisense construct (data not shown).

**DISCUSSION**

1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\) affects the growth and differentiation of numerous cell types (22–26). Relevant to this report HL-60 cells have been shown to differentiate into monocytes-macrophages (24) and osteoclast-like cells (23) upon exposure to 1,25-(OH)\(\textsubscript{2}\)D\(\textsubscript{3}\). Expression of several genes including c-myc, c-fos, and PKCs, PKC\(\beta\), and PKC\(\gamma\) are regulated prior to the appearance of the mature monocyte-macrophage phenotype (7–9, 28). c-myc gene expression is decreased, c-fos gene expression is transiently increased, and PKC levels are increased in HL-60 cells during the process of cellular differentiation (25, 29, 30). Considering the nature of these early events and the accepted importance of these gene products in cell signaling and growth, it is likely that regulation of these genes is critical for induced HL-60 cell differentiation.
Recent reports revealed that the 1,25-(OH)$_2$D$_3$ receptor (vitamin D receptor) is a substrate for PKC$_\beta$ and that phosphorylation of vitamin D receptor is important for controlling osteocalcin expression (3, 27). Such studies support and extend the possible roles PKCs have in modulating 1,25-(OH)$_2$D$_3$'s actions. Transcriptional response elements for 1,25-(OH)$_2$D$_3$ have also been identified. Interestingly, the response element for 1,25-(OH)$_2$D$_3$ in the osteocalcin gene contains a phorbol ester response element (31, 32). One factor that interacts with this AP-1 sequence is a heterodimer made up of c-fos and c-jun. PKC-directed phosphorylation of c-fos and c-jun regulates their AP-1 binding activity (33). However, the precise molecular nature of the interaction between the 1,25-(OH)$_2$D$_3$ signal transduction pathway and PKC for regulation of gene expression is still not clear. Several nuclear proteins have been shown to be phosphorylated by PKC during the course of myeloid cell differentiation (34). Our laboratory reported that 10 nuclear proteins undergo phosphorylation state changes within 6–40 h of 1,25-(OH)$_2$D$_3$ treatment (34). We identified several of these proteins as nuclear matrix or DNA packaging proteins, including several histones and lamin B. Therefore, PKCs act as regulators of nuclear events and may be intimately involved in the transduction of the 1,25-(OH)$_2$D$_3$ signal ultimately regulating gene expression and HL-60 cell differentiation.

Increasing evidence exists to indicate that PKC$_\beta$ plays an important role in 1,25-(OH)$_2$D$_3$ promotion of HL-60 cell differentiation. A variant HL-60 cell line (HL-525) lacking basal levels of PKC$_\beta$ is resistant to phorbol ester-induced differentiation (18). However, susceptibility to phorbol ester differentiation was restored if HL-525 cells were transfected to overexpress PKC$_\beta$. Additionally, phorbol 12-myristate 13-acetate resistance of HL-525 cells was reversed by pretreating with 1,25-(OH)$_2$D$_3$, which increased PKC$_\beta$ levels (18). Also, it was

**FIG. 5. Immunoflow cytometry analysis of CD14 surface antigen expression.** A, control versus 20 nM 1,25-(OH)$_2$D$_3$-treated cells. B, 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$ sense versus 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$ antisense. C, 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$II sense versus 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$II antisense. D, 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$II sense versus 20 nM 1,25-(OH)$_2$D$_3$, and 30 μM PKC$_\beta$ antisense. Cells were harvested after 48 h of treatment and incubated with fluorescein-labeled anti-CD14 antibody. The intensity of CD14 expression by individual cells are presented as histograms. This figure is representative of three independent experiments.

**FIG. 6. Effects of PKC$_\beta$II antisense on 1,25-(OH)$_2$D$_3$, inhibition of HL-60 cell proliferation.** HL-60 cells were treated with control, 20 nM 1,25-(OH)$_2$D$_3$, or 20 nM 1,25-(OH)$_2$D$_3$ in the presence of 30 μM PKC$_\beta$II sense or PKC$_\beta$II antisense. Cell number was determined by Coulter counter on the indicated days. Cell number was significantly ($p < 0.05$) different in control cells versus 1,25D$_3$ + βIIs or 1,25D$_3$ + βIIas at days 4, 5, and 6. No significant ($p > 0.05$) difference in cell number was observed between 1,25D$_3$ + βIIs and 1,25D$_3$ + βIIas over the entire time course.
shown that a 25-mer PKCβ antisense construct different from the one used here was capable of partially blocking (averaging ~30%) 1,25-(OH)2D3’s induction of cell differentiation (19). Although a partial inhibition of 1,25-(OH)2D3-promoted cell differentiation was observed using their antisense construct, it had little effect on 1,25-(OH)2D3 inhibition of cell proliferation. In our study, novel 15-mer PKCβ and PKCβII antisense constructs were found to inhibit 1,25-(OH)2D3 promotion of cell differentiation by 80–90%. However, these antisense oligonucleotides had no effect on 1,25-(OH)2D3’s ability to inhibit cell proliferation. Moreover, reduction of basal levels of PKCβ was not required for PKCβ antisense to inhibit 1,25-(OH)2D3 promotion of cell differentiation. This result suggests that blocking de novo synthesis of PKCβ is the mechanism of action for the antisense construct. We demonstrated that PKCβII is uniquely responsible for 1,25-(OH)2D3 promotion of cell differentiation. There is controversy as to whether PKCβII is expressed in HL-60 cells. In all reports, PKCβII levels in unstimulated HL-60 cells is significantly lower than PKCβII levels (19–21, 35). In this study, we failed to detect measurable levels of PKCβII. This finding is in agreement with several reports (20, 21). However, others have shown, using different antibodies or Northern blot analysis, that 1,25-(OH)2D3 increased PKCβII protein levels or mRNA levels (19, 35).

The findings reported here indicate that PKCβII specifically participates in the signal transduction mechanisms employed by 1,25-(OH)2D3 to promote HL-60 cell differentiation. Interestingly, we found a direct correlation between the quantitative lowering of PKCβII protein levels and the degree of induced differentiation. The correlation between the increased levels of PKCβ induced by 1,25-(OH)2D3 and the extent of cellular differentiation (7–9) suggest that there are not spare PKCβs in these cells. Thus, we suggest that the levels of PKCβ are stoichiometrically related to promotion of differentiation. This study provides clear and convincing evidence that promotion of cell differentiation and inhibition of cell proliferation are two distinct processes by 1,25-(OH)2D3 that can be disassociated by blocking the expression of a single gene, PKCβII. To date, several analogs of 1,25-(OH)2D3 have been developed that are selective at affecting calcium mobilization and promoting terminal cellular differentiation. Our study suggests that it may be possible to further separate the actions of 1,25-(OH)2D3 into its capacity to promote cellular differentiation versus its capacity to inhibit cell proliferation.

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