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1,25-Dihydroxyvitamin D_3 Targets PKC-βII but Not PKC-α to the Basolateral Plasma Membranes of Rat Colonocytes

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Prior studies by our laboratory have shown that 1,25-dihydroxyvitamin D_3 activated PKC-α, but not PKC-δ, -ϵ, or -ζ, in normal rat colonocytes. In the present studies we demonstrate for the first time that this secosteroid also activated PKC-βII, another DAG- and Ca^{2+}-dependent PKC isoform recently shown to be present in these cells. Moreover, this activation of PKC-βII by 1,25-dihydroxyvitamin D_3 treatment of isolated colonocytes was shown to be lost in cells from vitamin D-deficient rats and, at least partially, restored by repleting these animals with this secosteroid for 7 days. Under basal conditions, the expression of PKC-α and -βII in brush-border membranes was comparable to their respective expression in basolateral plasma membranes of rat colonocytes. In contrast, the expression of PKC-δ was significantly greater in brush-border membranes, whereas PKC-ϵ and -ζ were enriched in the basolateral plasma membranes. Furthermore, 1,25-dihydroxyvitamin D_3 specifically induced the translocation of PKC-βII, but not PKC-α, to the basolateral, but not brush-border plasma membranes of rat colonocytes, via a $pp60^{c\text{-}src}$-dependent mechanism.

Protein Kinase C (PKC) consists of a family of phospholipid-dependent, serine/threonine kinases, whose members include at least 12 isoforms that can be classified according to their co-factor requirements (1). Classical PKC isoforms (cPKCs) are regulated by both 1,2-diacylglycerol (DAG) and calcium and include isoforms α, βI, βII, and γ. Novel PKC isoforms (nPKCs) are activated by DAG, but are calcium-independent, and include PKC-δ, -ϵ, -η, -μ and -θ. Atypical isoforms (aPKCs) are both calcium- and DAG-independent and include ι, λ and ζ. These isoforms also differ in their tissue expression, substrate specificity and subcellular localization, which underlie the large number of cellular processes mediated by members of this family, albeit in isoform- and cell-specific manners (2-6).

Our laboratory has studied the roles of PKC isoforms in the regulation of important cellular processes in rat colonocytes (4, 5, 7) and in human colon cancer CaCo-2 cells (8-10). We have recently shown, for example, that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the major active metabolite of vitamin D_3, activated $pp60^{c\text{-}src}$, thereby inducing a significant increase in the basolateral plasma membrane (BLM) association, tyrosine phosphorylation and biochemical activity of PI-PLCγ (11). This, in turn, stimulated the hydrolysis of BLM, but not brush-border membrane (BBM) polyphosphoinositides, thereby generating inositol 1,3,4-trisphosphate (IP_3) and DAG (11). As a consequence of IP_3-mediated release of Ca^{2+} from intracellular stores (11, 12), and in conjunction with an influx of extracellular Ca^{2+} induced by this secosteroid (7, 8, 11, 12), intracellular Ca^{2+} ($[Ca^{2+}]_i$) was increased in these cells (7, 8, 12). This increase in $[Ca^{2+}]_i$, together with increased DAG levels, caused the transient (1-2 min) activation of PKC-α, but not other PKC isoforms present in these cells (7, 8). The activation of PKC-α, in turn, was shown to limit the $1,25(OH)_2D_3$-stimulated rise in $[Ca^{2+}]_i$ at a step distal to IP_3 generation in the BLM, most likely at the IP_3 receptor level, and did not affect the influx of intracellular Ca^{2+} into these cells (8).

In other studies our laboratory has also shown that the vitamin D status of rats markedly influenced the signal transduction events induced in colonocytes by the *in vitro* addition of $1,25(OH)_2D_3$ (13). In this regard, this secosteroid failed to activate all of the aforementioned biochemical events, including activation of PKC-

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Abbreviations used: PKC, protein kinase C; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; DAG, 1,2-diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; α, alpha; βI, beta-I; βII, beta-II; BBM, brush-border membrane; BLM, basolateral membrane; $[Ca^{2+}]_i$, intracellular calcium.

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α and increases in $[Ca^{2+}]_i$, in isolated colonocytes obtained from vitamin D-deficient rats (13). Moreover, each of these *in vitro* responses was, at least partially but significantly, restored after *in vivo* vitamin D-repletion (13).

In prior studies in rat colonocytes, we have demonstrated that these cells possess PKC-α, -δ, -ϵ and -ζ (7). Utilizing more recently available antibodies, we have found that these cells also express PKC-βII (4, 5). In the present studies it was, therefore, of interest to determine if $1,25(OH)_2D_3$ also activated this DAG- and Ca^{2+}-dependent PKC isoform; and, if so, whether the vitamin D status of rats influenced the *in vitro* secosteroid-induced activation of this isoform.

In other cell types (14, 15), PKC-α and -βII have been shown to mediate distinct processes induced by an agonist which, at least in part, have been attributed to differences in their subcellular localization (14). Moreover, as noted above, $1,25(OH)_2D_3$ induces several cell surface biochemical events which precede PKC-α activation and/or do not appear to be mediated by this PKC isoform. Based on these considerations, in the present studies we, therefore, also examined the effects of $1,25(OH)_2D_3$ on the subcellular localization of these two cPKC isoforms in rat colonocytes. The results of these experiments, as well as a discussion of these potential physiological significance, serve as the basis for the present report.

METHODS

Studies in colonocytes of vitamin D-sufficient, D-deficient, and D-repleted rats. Male Sprague-Dawley rats, initially weighing 50 g, were obtained as weanlings from partially vitamin D-deficient mothers and placed on either a vitamin D-sufficient or vitamin D-deficient diet as previously described in detail (7, 13). The latter group was housed in a facility devoid of fluorescent light. After 20 wk, one-half of the vitamin D-deficient rats received l0 ng/100 g body wt of $1,25(OH)_2D_3$ by mini-osmotic pumps implanted subcutaneously (2001-2002; Alza, Palo Alto, CA) for 7days, whereas the others received vehicle as described (7, 13). The rats in each of the three groups were sacrificed, their colons removed and colonocytes harvested as previously described (12). Their vitamin D status was assessed by assaying serum collected at sacrifice for $25(OH)D_3$ and $1,25(OH)_2D_3$ as described (7, 16). Isolated colonocytes from each group were treated with $1,25(OH)_2D_3$ or vehicle (ethanol) for the indicated times and then used to prepare soluble and particulate fractions as described (12). In other experiments, colonocytes isolated from vitamin D-sufficient rats were treated with $1,25(OH)_2D_3$ or vehicle for the indicated times and highly purified brush-border (BBM) and basolateral plasma membranes (BLM) then isolated as described by our laboratory (17). The BBM and BLM fractions were enriched 15-18 fold in the marker enzymes cysteine-inhibitable alkaline phosphatase and Na^+-K^+-ATPase, respectively, and, in agreement with prior studies (17), showed minimal cross contamination by the other plasma membrane or by intracellular organelles. In some experiments, colonocytes obtained from D-sufficient rats, were pretreated with PPI (100 μM, final concentration, 15 min), a selective inhibitor of the $pp60^{c\text{-}src}$ non-receptor tyrosine kinase family, provided by Pfizer Central Research (Groton, CT). The cells were then treated with $1,25(OH)_2D_3$ (10 nM, final concentration) or vehicle for 60 sec and antipodal plasma membranes prepared as described above.

Preparations were boiled in SDS-containing buffer and proteins quantified by amido black staining (4). Proteins (20 μg) from the SDS-treated samples were separated on SDS-PAGE using a 10% resolving gel, electroblotted and probed for specific PKC isoforms using monoclonal PKC-α antibodies (UBI, Inc., Lake Placid, NY) and polyclonal antipeptide antibodies to PKC-βI, -βII, -δ, -ϵ and -ζ (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described in detail by our laboratory (4). PKC isoforms were detected by xerography, using an enhanced chemiluminescence system, and xerograms quantified as previously described (4). Film exposure times were adjusted such that this chemiluminescence system was linear between 5 and 40 μg of total protein loaded (18).

Statistical analysis Results were expressed as means ± SEM. Data was analyzed using ANOVA (Dunnett's test), with values of $p < 0.05$ considered significant.

RESULTS

$1,25(OH)_2D_3$ Activates PKC-βII in Rat Colonocytes

As shown in Figure 1A and 1B, $1,25(OH)_2D_3$ (10 nM, final concentration) rapidly (30 sec) activated PKC-βII, as assessed by its translocation to the particulate fraction of colonocytes isolated from vitamin D-sufficient rats. PKC-βII translocation peaked by 30 sec (~2.5-fold compared with control) and was still significantly increased up to 90 sec, but by 15 min the particulate distribution of this isoform was no longer significantly different from that of cells treated with vehicle (Fig. 1A& B). In contrast to these findings, $1,25(OH)_2D_3$ failed to activate this PKC isoform in colonocytes isolated from vitamin D-deficient animals (Fig. 1C). The *in vivo* repletion of D-deficient animals with $1,25(OH)_2D_3$ for 7 days, however, partially restored the ability of $1,25(OH)_2D_3$ to activate PKC-βII in isolated colonocytes harvested from these animals (Fig. 1C).

$1,25(OH)_2D_3$ Translocates PKC-βII, but Not PKC-α, to the Basolateral Membrane of Rat Colonocytes

Under basal conditions, both the BBM and BLM of colonocytes isolated from vitamin D-sufficient rats were found to contain PKC-α, βII, -δ, -ϵ and -ζ (Table I). In unstimulated cells, the abundance of PKC-α and -βII in BBM were both comparable to their respective expression in BLM. In BBM the basal expression of PKC-δ, however, was significantly higher than in BLM, whereas the abundance of PKC-ϵ and -ζ was significantly lower in BBM than in BLM (Table I).

As shown in Figure 2A, treatment of isolated colonocytes with $1,25(OH)_2D_3$ (10 nM, final concentration), caused a significant increase in PKC-βII in the BLM, but not the BBM, whereas there was no significant change in PKC-α abundance in either antipodal plasma membrane of cells isolated from vitamin D-sufficient rats. Furthermore, pretreatment of these cells for 15 min with PP1 (100 μM, final concen-

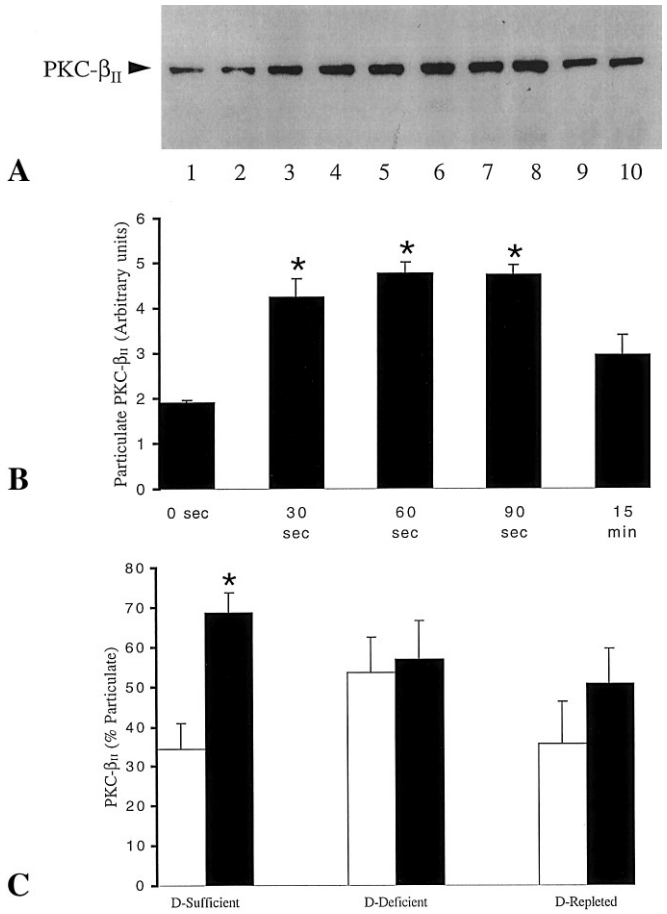

FIG. 1. Activation of PKC-β_{II} in rat colonocytes by 1,25(OH)$_2$D$_3$ and its modulation by vitamin D status. Colonocytes were isolated from vitamin D-sufficient or D-deficient rats, or vitamin D-deficient rats that were repleted *in vivo* with 1,25(OH)$_2$D$_3$ for 7 d as described under Methods. Cells were then treated with 10 nM 1,25(OH)$_2$D$_3$ or vehicle and subcellular fractions prepared and assayed for PKC-β_{II} as described under Methods. (**A**) PKC-β_{II} translocation. Cells from D-sufficient rats were treated at 37°C for 0 sec (lanes 1-2); 30 sec (lanes 3-4); 60 sec (lanes 5-6); 90 sec (lanes 7-8); 15 min (lanes 9-10). Shown is a Western blot of PKC-β_{II} in the particulate fractions of colonocytes treated in duplicate, representative of three independent experiments. (**B**) Time course of the 1,25(OH)$_2$D$_3$-induced translocation of PKC-β_{II} to the particulate fraction of D-sufficient rats. *p< 0.02. (**C**) Quantitative effect of vitamin D status on PKC β_{II} particulate association in response to 1,25(OH)$_2$D$_3$ (filled bars), or vehicle (open bars). Data in B and C are Means±SEM of 3 independent experiments. *p< 0.02, compared with vehicle-treated animals.

tration), a specific inhibitor of the pp60$^{c\text{-}src}$ non-receptor tyrosine kinase family (11), markedly reduced the ability of 1,25(OH)$_2$D$_3$ to translocate PKC-βII to the BLM (Fig. 2B).

DISCUSSION

The present studies demonstrate for the first time that 1,25(OH)$_2$D$_3$ activates PKC-βII in normal rat colonocytes. Like PKC-α, the DAG- and Ca^{2+}-dependence of this PKC isoform, its similar rapid, but transient response to 1,25(OH)$_2$D$_3$, as well as its dependence on pp60$^{c\text{-}src}$, suggest that PKC-βII is stimulated via the same secosteroid-mediated biochemical events noted earlier by our laboratory for PKC-α. Moreover, the

TABLE I

Distribution of PKC Isoforms in Antipodal Plasma Membranes of Rat Colonocytes[a]

PKC isoforms	BBM[b]	BLM[c]
α	98 ± 0.7	80.8 ± 13.3
βII	101.0 ± 3.2	105.2 ± 14.6
δ	94.5 ± 4.8	41.3 ± 4.2*
ϵ	92.0 ± 4.1	120.3 ± 6.5*
ζ	91.9 ± 5.9	209.4 ± 10.1*

[a] Values represent Means ± SEM in arbitrary densitometry units of three separate experiments.
[b] BBM, brush-border membranes.
[c] BLM, basolateral membranes.
* $p < 0.02$ compared to same PKC isoform values in BBM.

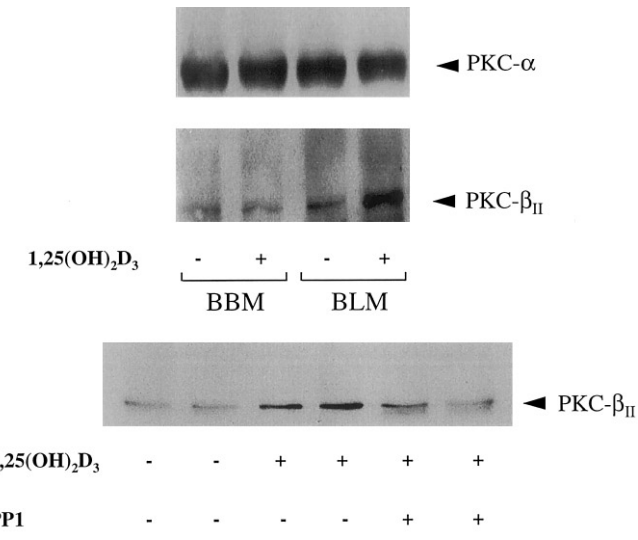

FIG. 2. Differential effects of 1,25(OH)$_2$D$_3$ on the particulate association of PKC-α and PKC-β_{II} to the antipodal plasma membranes of rat colonocytes. Colonocytes were prepared from D-sufficient rats and, after treatment with vehicle or 1,25(OH)$_2$D$_3$ (10 nM for 60s), the cells were lysed and brush border (BBM) or basolateral plasma membranes (BLM) prepared. Membrane-associated PKC-α and β_{II} isoforms were detected by Western blotting. (**A**) Representative Western blots of the effect 1,25(OH)$_2$D$_3$ on the association of PKC-α and PKC-β_{II} to the BLM and BBM. (**B**) Effect of pp60$^{c\text{-}src}$ inhibition on 1,25(OH)$_2$D$_3$ stimulated PKC-β_{II} translocation to the BLM. Isolated colonocytes were preincubated with PP1, a selective inhibitor of pp60$^{c\text{-}src}$ (100 μM for 15 min), and then treated with 1,25(OH)$_2$D$_3$ as described in A above. The cells were lysed and basolateral plasma membranes prepared. Western blots in A and B are representative of three independent experiments.

present observations with respect to the effects of the vitamin D status of rats on the activation of both PKC-α and -βII by 1,25$(OH)_2D_3$, lend additional support to this contention.

We have also now shown for the first time that in response to 1,25$(OH)_2D_3$, PKC-βII, but not PKC-α, translocated to the BLM, via a $pp60^{c\text{-}src}$-dependent mechanism, whereas neither of these two cPKC isoforms translocated to the BBM of these cells. These findings are in keeping with prior studies by our laboratory in which the activation of PKC-α by 1,25$(OH)_2D_3$ limited the secosteroid-induced increase in $[Ca^{2+}]_i$, by inhibiting the further release of Ca^{2+} from intracellular stores, presumably in the endoplasmic reticulum (8). They are also in keeping with the present observations that PKC-α failed to translocate to the BLM of these cells, since in these prior studies (8), PKC-α failed to inhibit the generation of IP_3 induced by 1,25$(OH)_2D_3$ in BLM. Given the transient nature of the 1,25$(OH)_2D_3$-stimulated BLM hydrolysis of polyphosphoinositides (1-2 min) in these cells (12), it would appear reasonable to suggest that the translocation of PKC-βII to this plasma membrane region of the rat colonocyte may serve as a negative-feedback mechanism to inhibit further generation of IP_3 and DAG by this secosteroid. This issue will, however, require further study.

In addition, the present observations are of interest with respect to the regulation of $[Ca^{2+}]_i$ and Ca^{2+} transport by 1,25$(OH)_2D_3$. In this regard, as noted previously, 1,25$(OH)_2D_3$ not only increased $[Ca^{2+}]_i$ via release of Ca^{2+} from intracellular stores, which was subsequently inhibited by PKC-α, but also increased $[Ca^{2+}]_i$ via a PKC-α-independent influx of extracellular Ca^{2+} across the plasma membranes(s) of these cells (8). Moreover, in vitamin D-deficient animals, this influx of extracellular Ca^{2+} in response to 1,25$(OH)_2D_3$ was abolished (13), concomitant with the loss of the ability of this secosteroid to activate PKC-βII.

In addition to these effects of 1,25$(OH)_2D_3$ on $[Ca^{2+}]_i$, this secosteroid has been shown to mediate the rapid (min) stimulation of transcellular Ca^{2+} transport in an *ex vivo* perfused intestinal system, which has been termed transcaltachia (19-21). This PKC-dependent process is only operative in the vitamin D-repleted chick and is not observed in the vitamin D-deficient chick intestine (19-21), and, moreover, is dependent on secosteroid-induced biochemical events which occurred in the BLM, but not the BBM of these cells. In this regard, it is of interest that recent studies have shown that PKC can modulate the activity of the 1,25$(OH)_2D_3$-regulated Ca^{2+} pump (22), located in the BLM of rat colonocytes. Taken together, these observation, therefore, indicate that the translocation of PKC-βII induced by 1,25$(OH)_2D_3$ may regulate the influx of extracellular Ca^{2+} into these cells and/or transcellular Ca^{2+} transport in rat colonocytes, although further studies will be necessary to address these issues.

Finally, while the present studies have demonstrated that 1,25$(OH)_2D_3$ failed to activate PKC-δ, -ϵ or -ζ in rat colonocytes, their basal expression was found to differ significantly in the antipodal plasma membranes of these cells. In future experiments it will, therefore, be of interest to determine the potential physiological significance of these differences in PKC isoform abundance in the BBM and BLM of rat colonocytes.

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