Journal of Cellular Biochemistry 96:97–108 (2005)

Regulation of Parathyroid Hypertensive Factor Secretion by Vitamin D_3 Analogs in Parathyroid Cells Derived From Spontaneously Hypertensive Rats

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Abstract Parathyroid hypertensive factor (PHF) is a novel substance secreted by the parathyroid gland (PTG), which is elevated in 30–40% of all hypertensive patients; specifically, the low-renin subset. However, very little is known about the regulation of PHF secretion. Since the classical parathyroid regulator, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), may be elevated concurrent with or preceding the development of low-renin hypertension and elevated plasma PHF, we hypothesized that 1,25-$(OH)_2D_3$ would stimulate PHF release. To test this hypothesis, PTG organ and cell cultures, derived from spontaneously hypertensive rats (SHR) and the normotensive genetic control Wistar Kyoto (WKY) rats, were exposed to various vitamin D_3 metabolites and PHF release measured by ELISA. 1,25-$(OH)_2D_3$ rapidly stimulated PHF release with enhanced sensitivity in SHR versus WKY cultures indicated by a leftward shift in the dose-response curve, whereas 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) had the converse effect. Vitamin D_3 analog ''BT,'' an agonist for the classical nuclear vitamin D receptor (1,25VDR_{nuc}), was without effect suggesting a 1,25VDR_{nuc}-independent mechanism and potential involvement of the plasma membrane-bound vitamin D receptor (1,25 D_3-MARRS). Interestingly, protein expression of the 1,25 D_3-MARRS was increased in SHR versus WKY parathyroid cells. In conclusion, these results support the idea that 1,25-$(OH)_2D_3$ may contribute to elevated plasma PHF in the SHR. J. Cell. Biochem. 96: 97–108, 2005.

Key words: 1,25-dihydroxyvitamin D_3; parathyroid hypertensive factor; 1,25 D_3-MARRS; nuclear vitamin D_3 receptor

The hormonally active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), in concert with parathyroid hormone (PTH), plays a critical role in plasma Ca^{2+} and phosphate homeostasis. Hypocalcemia, directly and indirectly via stimulation of PTH release, stimulates accumulation of 1,25-$(OH)_2D_3$ through activation of kidney 25-hydroxyvitamin D_3-1α-hydroxylase (1αOHase) and hydroxylation of the circulating precursor 25-hydroxyvitamin D_3 [DeLuca, 1974, 1979, 1988]. 1,25-$(OH)_2D_3$ functions to increase the pool of available plasma Ca^{2+} by stimulating absorption in the intestine, reabsorption in the kidney, and under low dietary calcium intake conditions, bone remodeling and resorption [DeLuca, 1974, 1979, 1988]. Part of the negative feedback control of this endocrine system occurs in the parathyroid gland (PTG), where 1,25-$(OH)_2D_3$ transcriptionally [Demay et al., 1992] suppresses PTH synthesis, inhibits PTH release, and inhibits parathyroid cell proliferation. In addition to the classical roles of 1,25-$(OH)_2D_3$, there are also many non-calcemic functions. Some of these include antiproliferative and pro-differentiative effects in promyelocyte HL-60 cells [Tanaka et al., 1982], keratinocytes [Hosomi et al., 1983], and vascular smooth muscle cells [Dokoh et al., 1983].

An extensive non-classical role for 1,25-$(OH)_2D_3$ has also been defined in the cardiovascular system. Amongst the cardiovascular effects of 1,25-$(OH)_2D_3$ are positive inotropism

Grant sponsor: NSERC; Grant sponsor: IPS Scholarship (to S.K.S.); Grant sponsor: MRC (to C.G.B.); Grant number: UI 13521.

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Received 19 November 2004; Accepted 5 April 2005
DOI 10.1002/jcb.20528

in cardiomyocytes [Jahn et al., 1991] and stimulation of L-type Ca^{2+} channel current and increased intracellular Ca^{2+} [Shan et al., 1993] in vascular smooth muscle cells. Abnormalities in both the cardiovascular function and the plasma levels of 1,25-$(OH)_2D_3$ have implicated a role for 1,25-$(OH)_2D_3$ in experimental and human hypertension. Plasma 1,25-$(OH)_2D_3$ has been shown to be elevated in low-renin and salt-sensitive human hypertension [Resnick, 1986] as well as in several rat models of low-renin hypertension [DiPette et al., 1990; Kotchen et al., 1990; Wu et al., 2000]. Furthermore, several studies have shown that chronic 1,25-$(OH)_2D_3$ administration potentiates induced contractility in vitro in vascular tissue [Hatton et al., 1994] and in mesenteric arteries taken from the SHR [Bukoski et al., 1989], but not from the genetic control strain, Wistar Kyoto (WKY) rats. Not surprisingly then, a role for 1,25-$(OH)_2D_3$ in blood pressure regulation in hypertension has been suggested [Tabuchi et al., 1986] and transient effects on systolic blood pressure in male hypertensive patients (but not in normotensives) have been observed [Jespersen et al., 1998]. However, chronic treatment with 1,25-$(OH)_2D_3$ was not shown to exert any significant effect on blood pressure in the SHR [Hatton et al., 1994].

Since 1,25-$(OH)_2D_3$ does not appear to have chronic effects on blood pressure, it has been suggested that 1,25-$(OH)_2D_3$ may modulate other endocrine systems, which produce more long-term hypertensive effects. Parathyroid hypertensive factor (PHF), a substance of parathyroid origin that may contribute to hypertension by potentiating the intracellular calcium-raising effects of classical vasoconstrictors, was thought to be a potential candidate for regulation by 1,25-$(OH)_2D_3$ (for review see Pang et al. [1996]). PHF is elevated in the same subset of hypertensives, that is, low-renin, salt-sensitive, that 1,25-$(OH)_2D_3$ is found to be elevated (for review see Pang et al. [1996]). As the PTG is a known target for 1,25-$(OH)_2D_3$, it was hypothesized that 1,25-$(OH)_2D_3$ would stimulate secretion of PHF.

We report here that 1,25-$(OH)_2D_3$ indeed stimulates PHF release rapidly and sub-acutely with enhanced sensitivity in parathyroid cells derived from SHR relative to the normotensive control strain. Conversely, the metabolite 24,25-$(OH)_2D_3$ was shown to rapidly and sub-acutely inhibit PHF release with blunted sensitivity in cells derived from SHR. Furthermore, by utilizing the 1,25VDR_{nuc}-specific agonist analog BT (1,24$(OH)_2$-22-ene-24-cyclopropyl-D_3), which is incapable of inducing the rapid effects of 1,25-$(OH)_2D_3$ observed in some systems [Farach-Carson et al., 1991], we will provide evidence suggesting the rapid effect of 1,25-$(OH)_2D_3$ on PHF release, which may be mediated by the newly described membrane-associated, rapid-response steroid-binding protein 1,25 D_3-MARRS.

MATERIALS AND METHODS

Materials

Mouse anti-PHF oligoclonal antibody (IgM-3A), standard semi-pure PHF, and PHF-horseradish peroxidase conjugate (PHF-HRP) were prepared as described previously [Krylova et al., 2000]. Monoclonal anti-1,25VDR_{nuc} IgG (clone 9A7) and recombinant nuclear Vitamin D receptor protein were purchased from Affinity Bioreagents, Inc. (Golden, CO), and monoclonal anti-Histone H1 IgG (clone AE-4) was purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Anti-1,25 D_3-MARRS antisera Ab99 was generated as described previously [Nemere et al., 1994, 1998]. Vitamin D_3 analogs 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 were obtained from Roussell UCLAS and Sigma Aldrich Chemical Company (St. Louis, MO), and 1,24$(OH)_2$-22-ene-24-cyclopropyl-D_3 (BT) was a generous gift from Leo Pharmaceuticals (Copenhagen, Denmark). All cell culture media were obtained from Gibco/BRL (Burlington, ON, Canada). BCA protein assay reagents were obtained from Pierce Chemical Company (Rockford, IL) and acrylamide, bis, temed, ammonium persulfate, and kaleidoscope molecular weight standards were all purchased from BioRad (Hercules, CA). Polyvinylidene difluoride (PVDF) membranes (Immobilon-P) were obtained from Millipore (Bedford, MA) and enhanced chemiluminescence (ECL) detection reagents for Western blot analysis were obtained from Amersham (Arlington Heights, IL). WST-1 cell proliferation reagent was obtained from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals were purchased from either Sigma/Aldrich (St. Louis, MO) or Fisher chemicals (Fairlawn, NJ). SHR and WKY rats were obtained from Charles River (St. Constant, Quebec, Canada).

PHF Secretion in Response to Vitamin D_3 Analogs

For investigation of the effects of different vitamin D_3 analogs on PHF secretion, both parathyroid cell and organ culture models were employed. Parathyroid cell suspensions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS were obtained as previously described [Benishin et al., 1999], seeded in 96-well plates for approximately 48 h to achieve a final cell density of 1.0×10^4 cells/well. Fresh solutions of vitamin D_3 analogs were prepared in ethanol, followed by dilution in culture medium (Ham's F12 adjusted to 1.5 mM Ca^{2+} and supplemented with 5% FBS) so that the final concentration of ethanol was less than 0.05% (v/v). Media were then gently aspirated and replaced with media containing vitamin D_3 analog or vehicle alone and incubated for either 30 min or 48 h at 37°C. Aliquots of 20–50 μl/well were then collected and processed for PHF analysis as previously described [Sutherland and Benishin, 2004], media aspirated and replaced with phenol red-free fresh culture medium (100 μl/well), and analyzed for cell viability as described below. For experiments involving parathyroid organ culture, glands were isolated from SHR and WKY rats as described previously [Benishin et al., 1999] and placed in 24-well culture dishes (2 glands/well) containing 250 μl/well culture medium containing 1,25-$(OH)_2D_3$ prepared as described above. Aliquots of 10 μl were removed at various time points for PHF detection [Sutherland and Benishin, 2004]. Use of animals was according to CCAC guidelines, and reviewed and approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Sub-Cellular Fractionation and Western Blots for 1,25 D_3-MARRS and 1,25VDR_{nuc} Protein

Parathyroid cells cultured in 150×10 mm tissue culture dishes were washed twice with Hank's balanced salt solution (HBSS) and 400 μl of a lysis buffer (15 mM Tris, 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, pH 7.4) was added. For whole cell extracts, the lysis buffer also contained 0.5% NP-40 and the cell lysate was harvested and frozen at −70°C. To obtain sub-cellular fractions, cell lysate was homogenized and sonicated for 5 min, then nuclei and cell debris were pelleted by centrifugation at 750*g* for 5 min and reconstituted with lysis buffer containing 0.5% NP-40 after the supernatant was collected. The supernatant was ultracentrifuged for 30 min at 100,000*g*, 4°C and the resulting supernatant representing the cytosolic fraction was decanted and the plasma membrane pellet washed two times with PBS and solubilized with lysis buffer containing 0.5% NP-40. Aliquots of all three sub-cellular fractions were frozen at −70 C until Western blot analysis. Protein content of the samples was measured using the BCA protein assay (Pierce) and samples were diluted in reducing treatment sample buffer and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gels as previously described by Laemmli [1970]. Proteins were transferred to a PVDF membrane by electroblotting at 75 V for 1.5 h in transfer buffer (19 mM Tris-HCl, 150 mM glycine, 20% methanol), and immunodetection of specific bands was carried out according to the Western blotting protocol supplied by Millipore with the PVDF membranes. Primary antibody concentrations were 1:1,000 for rabbit anti-1,25 D_3-MARRS IgG (Ab99), 1:1,000 for rat anti-1,25VDR_{nuc} IgG (9A7), or 1:500 mouse anti-histone H1 (AE-4). All appropriate secondary antibodies were used at a dilution of 1:2,000, and specific proteins were visualized using the ECL detection system.

Statistical Analysis

ELISA standard curves and unknown values were determined using the Microplate Manager/PC software (BioRad Laboratories). Results are expressed as the mean ± SEM, and statistical evaluation for differences between treatment groups was performed using either *t*-tests or ANOVA (Student–Newman–Keuls test) where appropriate. $P < 0.05$ was taken to be significant. EC_{50}s were calculated using Graphpad Prism software, using non-linear regression fit with variable Hill slope.

RESULTS

Effect of 1,25-$(OH)_2D_3$ on PHF Secretion

The rapid and sub-acute effects of 1,25-$(OH)_2D_3$ on PHF release from cultured parathyroid cells are shown in Figure 1. Exposure to 1,25-$(OH)_2D_3$ for 30 min resulted in a dose-dependent stimulation of PHF release from parathyroid cells derived from both SHR and WKY (Fig. 1a) rats. The dose-response curves

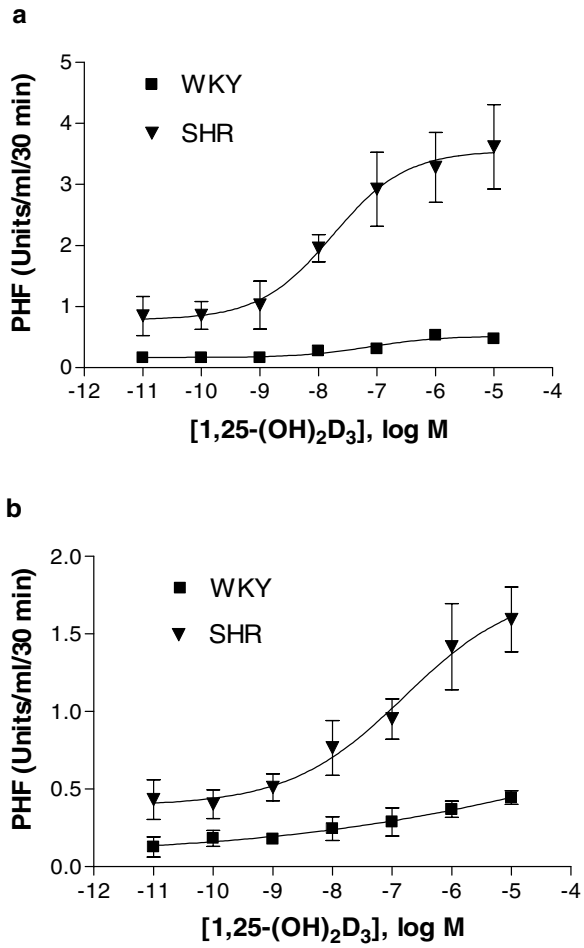

Fig. 1. Effect of 1,25-$(OH)_2D_3$ on PHF secretion from subcultured SHR (▼) and WKY (■) parathyroid cells. For investigation of the effect of 1,25-$(OH)_2D_3$ on cumulative PHF release after 30 min (**a**) and 48 h (**b**), parathyroid cell suspensions in DMEM supplemented with 5% FBS were seeded in 96-well plates at a density of approximately 1×10^4 cells/well. After a 48-h attachment period, medium was gently aspirated and replaced with 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS containing the indicated concentrations of 1,25-$(OH)_2D_3$ or ethanol vehicle (control) and incubated for 30 min (a) or 48 h (b) at 37°C. Aliquots of 50 µl/well were then collected and processed for PHF analysis, media aspirated and replaced with fresh 1.5 mM Ca^{2+} Ham's F12 medium plus 5% FBS (100 µl/well) and analyzed for cell viability. Each point is the mean ± SEM of three experiments, with at least six replicate wells per treatment group for each experiment.

followed a sigmoidal pattern and the SHR parathyroid cells displayed an apparent increase in sensitivity to 1,25-$(OH)_2D_3$ as indicated by the lower EC_{50} [1.76×10^{-8} M (Hill slope = 0.697, $R^2 = 0.99$) for SHR, vs. 8.04×10^{-8} M (Hill slope = 0.547, $R^2 = 0.93$) for WKY]. The maximal stimulation elicited by 1,25-$(OH)_2D_3$ was also greater in SHR parathyroid cells. Extension of the incubation time to 48 h also resulted in dose-dependent stimulation of PHF secretion by 1,25-$(OH)_2D_3$, and the enhanced apparent sensitivity of SHR parathyroids was maintained (Fig. 1b) [EC_{50}s: 1.59×10^{-7} M (Hill slope = 0.452, $R^2 = 0.99$) for SHR, vs. 6.47×10^{-4} M (Hill slope = 0.155, R^2 = ND) for WKY].

Figure 2 shows the effect of 1,25-$(OH)_2D_3$ on PHF release from intact PTG organ culture. 1,25-$(OH)_2D_3$ stimulated PHF secretion from SHR and WKY PTG in a similar manner as for cultured parathyroid cells. PTGs were rapidly stimulated by 1 µM 1,25-$(OH)_2D_3$ to secrete PHF after 4 h exposure for both SHR and WKY PTG cultures (Fig. 2a). However after 48 h, although mean PHF was increased, 1 µM 1,25-$(OH)_2D_3$ did not significantly stimulate PHF release for SHR and WKY PTG cultures (Fig. 2b).

Taken together, these results indicate that 1,25-$(OH)_2D_3$ rapidly and sub-acutely stimulates PHF release from parathyroid cells and that sensitivity is enhanced in parathyroid cells derived from SHR animals.

Effect of 24,25-$(OH)_2D_3$ on PHF Secretion

To determine whether effects of 1,25-$(OH)_2D_3$ on PHF release were vitamin D_3 analog specific, the effect of 24,25-$(OH)_2D_3$ on basal PHF secretion was examined. In contrast to 1,25-$(OH)_2D_3$, exposure to 24,25-$(OH)_2D_3$ for 30 min resulted in a dose-dependent inhibition of basal PHF release from parathyroid cells derived from both SHR (Fig. 3a) and WKY (Fig. 3b) rats. The SHR PTC dose-response curve followed a sigmoidal pattern with an IC_{50} of 5.06×10^{-9} M, however the WKY PTC dose-response curve revealed an unusual logarithmic relationship between 24,25-$(OH)_2D_3$ concentration and PHF secretion, which indicated increased sensitivity to 24,25-$(OH)_2D_3$ relative to the SHR PTC. Extension of the incubation time to 48 h resulted in maintained dose-dependent inhibition of PHF secretion by 24,25-$(OH)_2D_3$, although at this point, the dose-response curve for SHR PTC (Fig. 3c) displayed the unusual logarithmic pattern, and the WKY PTC (Fig. 3d) dose response curve followed the expected sigmoidal pattern and yielded an IC_{50} of 4.70×10^{-10} M.

Taken together, these results indicate that 24,25-$(OH)_2D_3$ rapidly and sub-acutely inhibits basal PHF release from parathyroid cells and

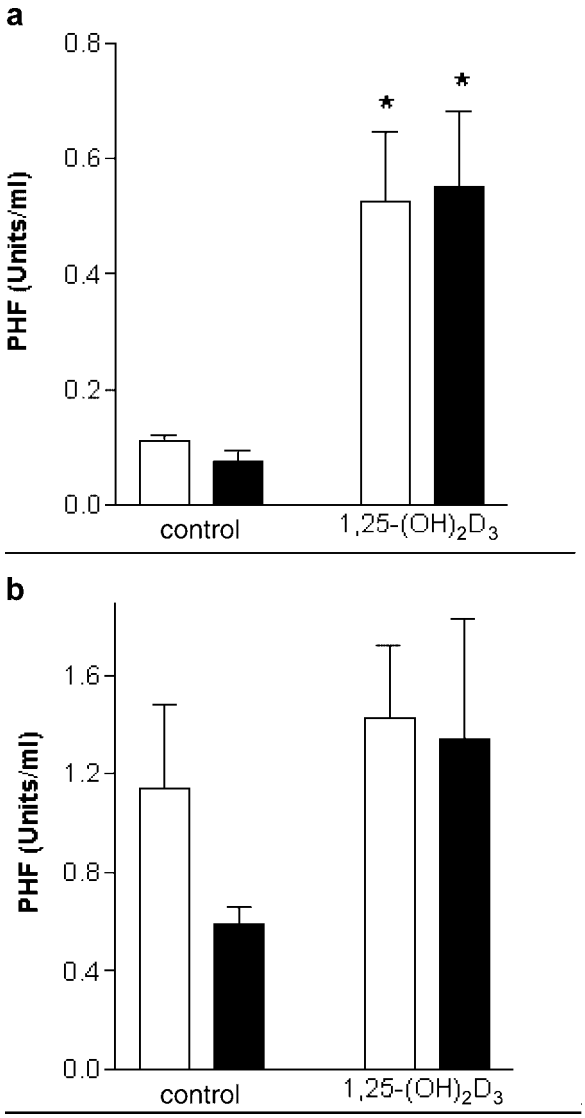

Fig. 2. Effect of 1,25-$(OH)_2D_3$ on PHF release in SHR and WKY parathyroid organ culture. Parathyroids were harvested from SHR (□) and WKY (■) rats and placed in 24-well culture plates (two glands/well) containing 1 μM 1,25-$(OH)_2D_3$ or ethanol vehicle (control) in 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS. Aliquots were taken after 4 h (**a**) and 48 h (**b**) and assayed for PHF by ELISA. Data points represent mean PHF ± SEM of four culture wells representing four animals for each of the experimental conditions. *Indicates significantly different versus control group ($P < 0.05$; *t*-test).

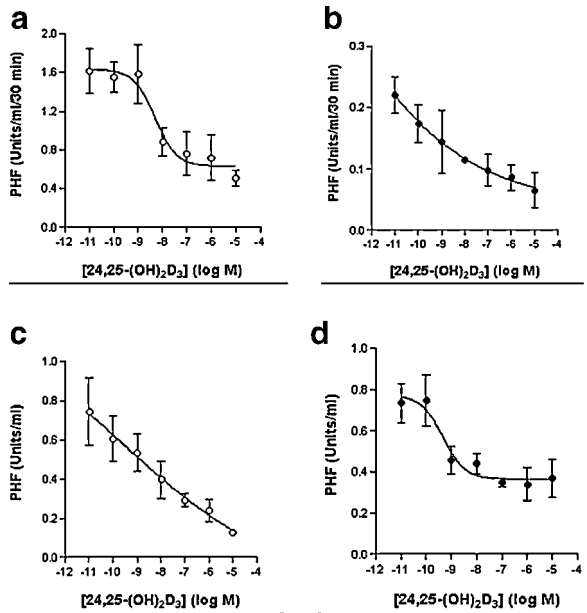

Fig. 3. Effect of 24,25-$(OH)_2D_3$ on PHF secretion from subcultured SHR and WKY parathyroid cells. For investigation of the effect of 24,25-$(OH)_2D_3$ on cumulative PHF release after 30 min (**a**, **b**) and 48 h (**c**, **d**), parathyroid cell suspensions in DMEM supplemented with 5% FBS were seeded in 96-well plates at a density of approximately 1×10^4 cells/well. After a 48-h attachment period, medium was gently aspirated and replaced with 1.5 mM Ca^{2+} Ham's F12 supplemented with 5% FBS containing the indicated concentrations of 24,25-$(OH)_2D_3$ or ethanol vehicle (control) and incubated for 30 min or 48 h at 37°C. Aliquots of 50 μl/well were then collected and processed for PHF analysis, media aspirated and replaced with fresh 1.5 mM Ca^{2+} Ham's F12 medium plus 5% FBS (100 μl/well) and analyzed for cell viability. Results shown are for SHR (a, c) and WKY (b, d) parathyroid cells and are presented as the mean ± SEM of three experiments with at least six replicate wells per treatment group for each experiment.

that sensitivity may be decreased in parathyroid cells derived from SHR animals.

VDR Protein Expression

Because parathyroid cells derived from either SHR or WKY rats displayed differential regulation of PHF release by 1,25-$(OH)_2D_3$, we examined the parathyroid expression of the classical 1,25VDR_{nuc} as well as 1,25 D_3-MARRS. Figure 4a shows 1,25 D_3-MARRS protein expression, indicated by a single band at approximately 66 kDa, is enhanced in subcultured SHR parathyroid cells versus cells derived from the normotensive strain, WKY. However, WKY parathyroid cells were shown to have slightly increased or equivalent levels of 1,25VDR_{nuc} protein versus SHR parathyroid cells (Fig. 4b). Further characterization of 1,25 D_3-MARRS in SHR and WKY PTC, according to sub-cellular localization, is shown in Figure 5a. The 1,25 D_3-MARRS seemed to represent a similar proportion of total protein in nuclear,

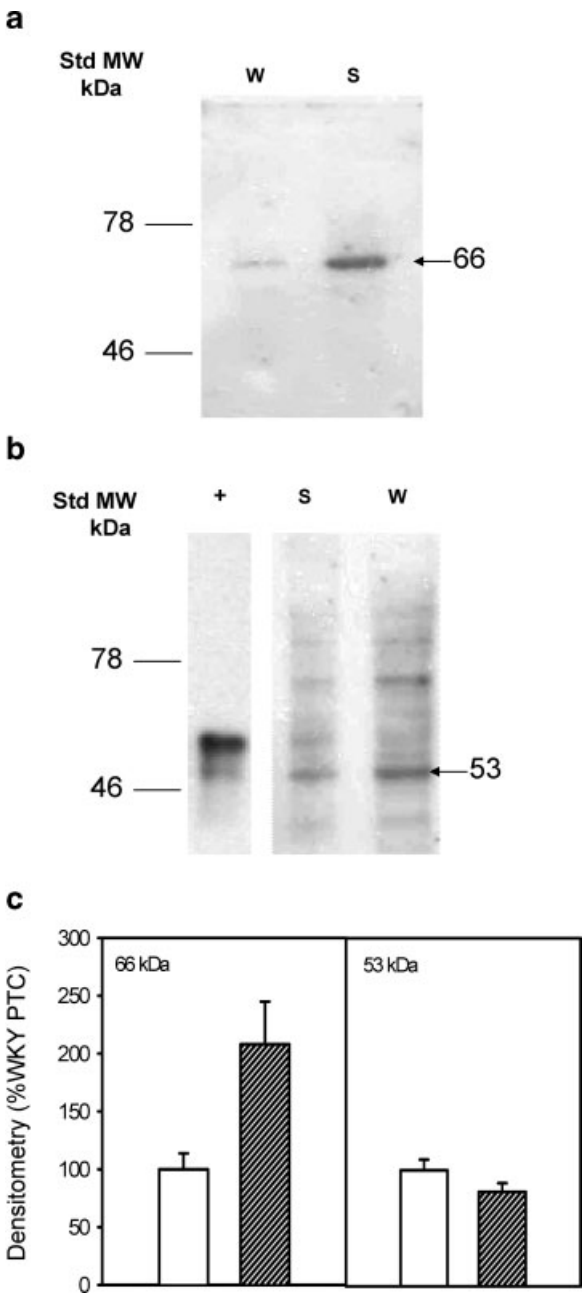

Fig. 4. 1,25 D_3-MARRS and 1,25VDR_{nuc} protein in SHR and WKY parathyroid cells. Western blots for 1,25 D_3-MARRS (**a**) and 1,25VDR_{nuc} (**b**) were performed on 1.25 μg of whole parathyroid cell extracts for both SHR (S) and WKY (W) parathyroid cells and positive (+) control purified recombinant 1,25VDR_{nuc} (10 ng) electrophoresed by SDS–PAGE on 10% gels. **c**: Densitometric analysis of the results presented in (a, 66 kDa band) and (b, 53 kDa band). The open bars are parathyroid extract from WKY rats, and the shaded bars are parathyroid extract from SHR. The results are normalized to the density for WKY PTC, and are the mean ± SEM for four separate gels.

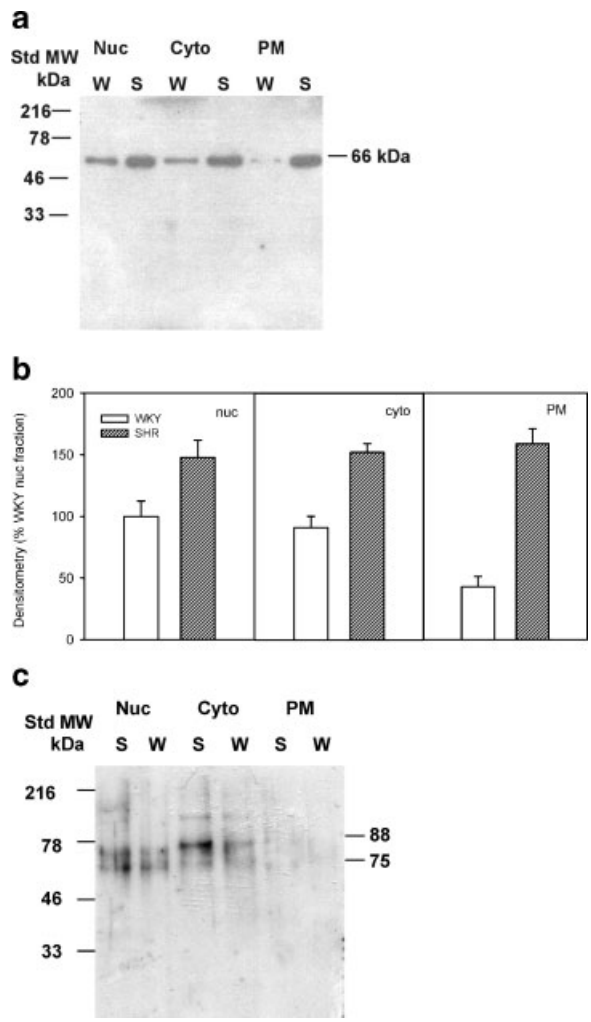

Fig. 5. Sub-cellular distribution of 1,25 D_3-MARRS in parathyroid cells. Cells were seeded at an intermediate density and allowed to attach for 48 h DMEM containing 5% FBS (until approximately 60–70% confluent) before cell lysates were obtained and subjected to sub-cellular fractionation. Shown are nuclear (Nuc), cytosolic (Cyto), and cell plasma membrane (PM) fractions (3 μg/lane) derived from SHR (S) and WKY (W) parathyroid cells, which have been analyzed for either 1,25 D_3-MARRS protein (**a**) or the nuclear marker, histone H1 (**c**), by Western blotting. **b**: Densitometric analysis of the results presented in (a, 66 kDa band), for the nuclear (nuc), cytoplasmic (cyto), and plasma membrane (PM) enriched fractions. The open bars are fractions prepared from WKY rats, and the shaded bars are fractions prepared from SHR. The results are normalized to the density for WKY nuclear fraction for comparison, and are the mean ± SEM for three separate gels.

cytosolic, and plasma membrane fractions prepared from whole parathyroid cell lysates for both SHR and WKY PTC except for a reduction in band intensity for the plasma membrane fraction of WKY PTC. Figure 5c shows that the

sub-cellular distribution of the nuclear marker, Histone H1, is nuclear and cytosolic indicating the plasma membrane fraction is largely free of nuclear protein contaminants.

Earlier studies have reported that the Ab99 does not cross-react with recombinant $1,25VDR_{nuc}$ [Nemere et al., 2000]. We have confirmed this observation by running duplicate samples and purified $1,25VDR_{nuc}$ on 8% SDS–PAGE gels and Western blotting half of the blot with anti-$1,25VDR_{nuc}$ (9A7) and the other to anti-1,25 D_3-MARRS (Ab99). The result is shown in Figure 6. Antibody 9A7 was shown to recognize two bands, one at approximately 66 kDa and another at the expected MW of 53 kDa. Probing with the anti-1,25 D_3-MARRS antisera resulted in only a 66 kDa band visible. Excess pure recombinant $1,25VDR_{nuc}$ was not recognized by Ab99. These data suggest that the 1,25 D_3-MARRS and $1,25VDR_{nuc}$ are of different molecular weights and that Ab99 recognizes an epitope of the 1,25 D_3-MARRS, which is either not present or is masked in the $1,25VDR_{nuc}$.

Role of the VDRs in Mediating the Effects of $1,25\text{-}(OH)_2D_3$ on PHF Secretion

To examine the extent of involvement of the 1,25 D_3-MARRS and classical $1,25VDR_{nuc}$ in mediating the effects of $1,25\text{-}(OH)_2D_3$ on PHF release, the vitamin D_3 analog $1,24(OH)_2$-22-ene-24-cyclopropyl-D_3 (BT) was used. This vitamin D_3 analog has high affinity for the $1,25VDR_{nuc}$, but was shown to be ineffective in stimulating a variety of rapid, non-genomic effects in other systems [Farach-Carson et al., 1991]. Exposure of SHR and WKY PTC to 100 nM BT had no effect on PHF release after either 30 min (Fig. 7a) or 48 h (Fig. 7b) in

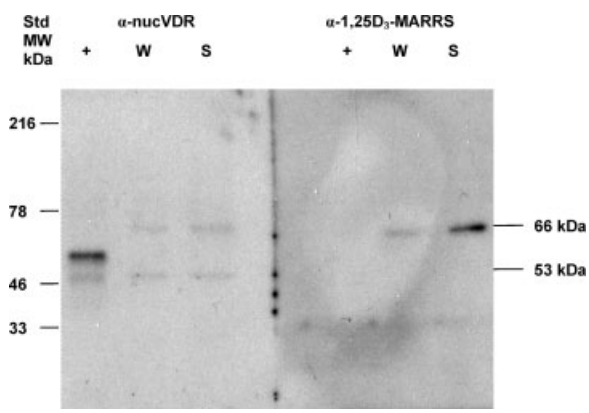

Fig. 6. Comparison of 1,25 D_3-MARRS and $1,25VDR_{nuc}$. Parathyroid cells derived from both SHR (S) and WKY (W) were seeded at either at an intermediate density and allowed to attach for 48 h in DMEM containing 5% FBS under approximately 60–70% confluent. Cell lysates were then collected and run on the same gel (1.25 μg), along with positive (+) control purified recombinant $1,25VDR_{nuc}$ (10 ng) in duplicate and one half of the gel analyzed for 1,25 D_3-MARRS protein and the other half for $1,25VDR_{nuc}$ protein by Western blotting. Result shown is a representative blot of three identical experiments yielding similar results.

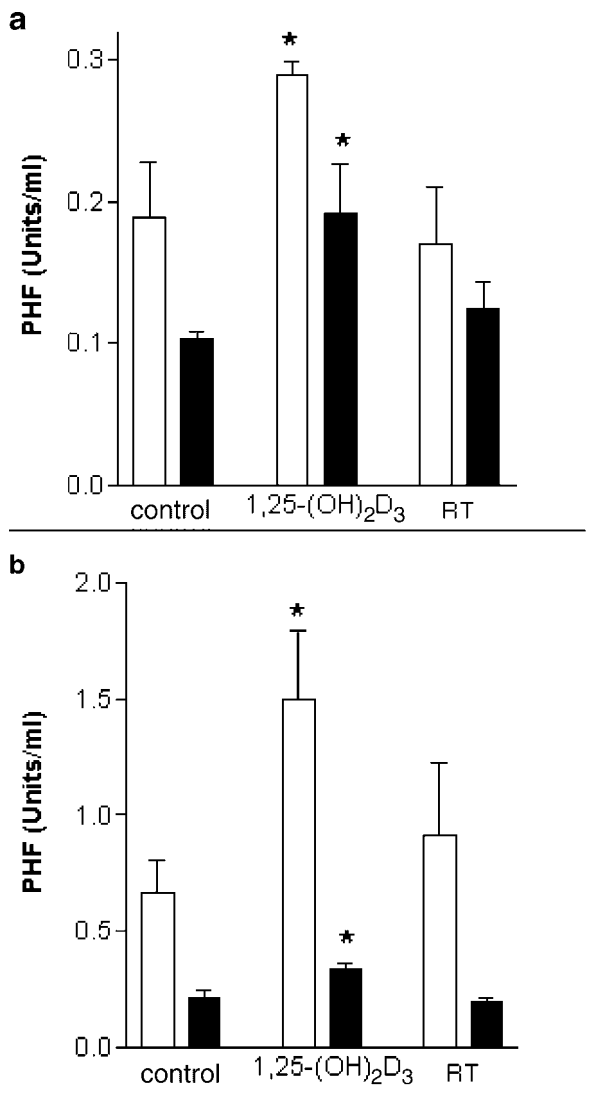

Fig. 7. Role of the $1,25VDR_{nuc}$ in mediating effects of $1,25\text{-}(OH)_2D_3$ on PHF release. Parathyroid cells derived from SHR (□) and WKY (■) rats were exposed to 100 nM $1,25\text{-}(OH)_2D_3$ ($1,25D_3$), 100 nM analog BT (BT), or ethanol vehicle alone (control) for 30 min (**a**) or 48 h (**b**) and media samples collected for PHF analysis in ELISA. Results shown indicate the mean PHF ± SEM for three separate experiments. *Indicates significantly different from the control group for the same rat strain ($P < 0.05$; *t*-test).

culture. These results strongly suggest that the $1,25VDR_{nuc}$ is not involved in mediating the rapid and sub-acute effects of $1,25\text{-}(OH)_2D_3$ on PHF secretion from SHR or WKY parathyroid cells.

DISCUSSION

In this study, the role of different vitamin D_3 analogs in regulation of secretion of PHF from parathyroid tissue from both normotensive and hypertensive rats was examined. $1,25\text{-}(OH)_2D_3$ was considered an obvious candidate hypothesized to directly stimulate PHF secretion based on the plasma profile of elevated $1,25\text{-}(OH)_2D_3$ and PHF in low-renin hypertension, and the classical role of $1,25\text{-}(OH)_2D_3$ in regulation of the PTG. In addition, parathyroid cells derived from the low-renin hypertensive model, SHR, were predicted to display enhanced sensitivity to $1,25\text{-}(OH)_2D_3$. This study characterized the rapid and sub-acute stimulatory effects of $1,25\text{-}(OH)_2D_3$ and inhibitory effects of $24,25\text{-}(OH)_2D_3$ on PHF release and revealed that SHR parathyroid cells displayed enhanced sensitivity to $1,25\text{-}(OH)_2D_3$ and reduced sensitivity to $24,25\text{-}(OH)_2D_3$. This is also the first demonstration of the parathyroid expression of the newly described 1,25 D_3-MARRS. Furthermore, PHF secretion studies using the classical $1,25VDR_{nuc}$ agonist BT revealed that the $1,25VDR_{nuc}$ was not likely to be mediating the stimulatory effects of $1,25\text{-}(OH)_2D_3$ on PHF release, leaving open the possibility of 1,25 D_3-MARRS mediating these rapid effects in the parathyroid. These data support the idea that vitamin D_3 metabolites and altered sensitivity to them with respect to PHF secretion contribute to the elevated PHF status of the SHR.

The original focus of this work was to examine and characterize the potential role of the biologically active vitamin D_3 metabolite, $1,25\text{-}(OH)_2D_3$, in regulation of PHF release. Both parathyroid cultured cells and intact glands were more responsive to rapid regulation, as opposed to sub-acute regulation, of PHF secretion by $1,25\text{-}(OH)_2D_3$. In SHR parathyroid cell cultures, enhanced sensitivity to $1,25\text{-}(OH)_2D_3$ was observed at 30 min versus the 48 h time point suggesting that the primary effect of $1,25\text{-}(OH)_2D_3$ on PHF release occurs rapidly and that the lingering effects of this rapid release are maintained by 48 h. Conversely, sensitivity to inhibition of PHF release by $24,25\text{-}(OH)_2D_3$ was unchanged between 30 min versus 48 h for SHR parathyroid cells; however, WKY parathyroid cells displayed increased sensitivity after 30 min versus 48 h exposure to $24,25\text{-}(OH)_2D_3$. Similar results were observed for parathyroid organ cultures with significant effects of $1,25\text{-}(OH)_2D_3$ after 4, but not 48 h. Other regulators, such as extracellular calcium [Sutherland and Benishin, 2004], have also been shown to exert only rapid effects on PHF release, which suggests that PHF may be stored, and that regulation of release largely occurs post-transcriptionally through modulation of the secretory pathway rather than by altering PHF synthesis.

Rapid effects of $1,25\text{-}(OH)_2D_3$ (and other steroids) have been described in many systems and cancerous cell lines and is reviewed elsewhere [Norman, 1998]. In parathyroid cells, rapid responses to $1,25\text{-}(OH)_2D_3$, such as increased cytosolic Ca^{2+} [Sugimoto et al., 1988] and phospholipid metabolism [Bourdeau et al., 1990], have been described. It is possible that these effects may be mediated by the classical nuclear vitamin D_3 receptor, which is expressed in PTG [Henry and Norman, 1975; Hughes and Haussler, 1978] and maintained in sub-cultured parathyroid cells [Brown et al., 1992]. However, it is more likely that 1,25 D_3-MARRS, which has been shown to mediate other rapid effects of $1,25\text{-}(OH)_2D_3$ such as stimulation of PKC and antiproliferative effects in growth zone chondrocytes [Nemere et al., 1998; Pedrozo et al., 1999], and stimulated phosphate uptake in intestinal epithelium [Nemere et al., 2004] is involved. The effects of $1,25\text{-}(OH)_2D_3$ on parathyroid cytosolic Ca^{2+} were insensitive to either diltiazem or verapamil, which led the authors to conclude that $1,25\text{-}(OH)_2D_3$ may be affecting Ca^{2+} entry through direct interaction with the cell plasma membrane and not through modulation of voltage-sensitive Ca^{2+} channels [Sugimoto et al., 1988]. This suggests that a receptor-operated Ca^{2+} channel, possibly involving 1,25 D_3-MARRS, may be involved in mediating the rapid $1,25\text{-}(OH)_2D_3$-induced Ca^{2+} entry.

To eliminate the possibility that the $1,25VDR_{nuc}$ is involved in regulation of PHF secretion, we employed the vitamin D_3 analog BT, a classical $1,25VDR_{nuc}$ agonist that is incapable of exerting the rapid effects of $1,25\text{-}(OH)_2D_3$ observed in some systems [Farach-Carson et al., 1991]. It is not known if BT can bind 1,25 D_3-MARRS or act as an agonist for this receptor, however the lack of effect of BT on PHF

release suggests a 1,25VDR$_{nuc}$-independent mechanism of action for 1,25-$(OH)_2D_3$ regulation of PHF secretion. This leaves open the possibility of 1,25 D_3-MARRS for transduction of these rapid effects.

Parathyroid cells derived from SHR appeared to display increased sensitivity to stimulation of PHF secretion by 1,25-$(OH)_2D_3$ after 30 min versus cells derived from the normotensive WKY strain. There are several possible explanations for either differences in maximal response or sensitivity to 1,25-$(OH)_2D_3$ between the strains with respect to PHF release. Enhanced sensitivity in SHR parathyroid cell cultures may indicate altered post-vitamin D_3 receptor signal transduction pathways. Western blot analysis showed increased 1,25 D_3-MARRS in SHR parathyroid cells compared to WKY, but no differences or even slightly decreased expression of the 1,25VDR$_{nuc}$ protein. This may account for the increased PHF secretory response to 1,25-$(OH)_2D_3$ in SHR parathyroid cells if 1,25 D_3-MARRS, in fact, mediates the stimulatory effects of 1,25-$(OH)_2D_3$ on PHF release. If this expression pattern is also observed in vivo, this could at least partly account for the elevated plasma PHF observed in SHR. Earlier 1,25-$(OH)_2D_3$ binding studies showed increased maximal specific binding capacity for calcitriol in SHR parathyroid tissue suggesting increased receptor expression [Merke et al., 1989]. Since we have shown that expression of the 1,25VDR$_{nuc}$ is unchanged between strains or slightly reduced for SHR parathyroid cells, it is likely that the enhanced binding they observed was due to 1,25 D_3-MARRS. A second explanation for enhanced sensitivity to 1,25-$(OH)_2D_3$ in the SHR parathyroid may be due to a prolonged half-life due to either alterations in 1,25-$(OH)_2D_3$ release from vitamin D_3 binding proteins or decreased activity of cytochrome P450 hydroxylases, which are known to metabolically inactivate free 1,25-$(OH)_2D_3$ in parathyroid cells [Brown et al., 1992]. A final possibility is that alterations in the calcitriol receptor involved in mediating these effects or the downstream signaling pathways, if the effects are indeed receptor-mediated, may be responsible for enhanced sensitivity of SHR parathyroid tissue to 1,25-$(OH)_2D_3$.

The observation that 1,25 D_3-MARRS, but not the 1,25VDR$_{nuc}$, was differentially expressed in the SHR versus WKY parathyroid, which led us to investigate some of the characteristics of 1,25 D_3-MARRS. Western blotting analysis indicated that 1,25 D_3-MARRS is slighter larger than the 1,25VDR$_{nuc}$ (66 kDa vs. 53 kDa). Furthermore, as previously reported [Pedrozo et al., 1999; Nemere et al., 2000], anti-1,25 D_3-MARRS antisera does not cross-react with excess pure 1,25VDR$_{nuc}$ indicating the antibody recognizes an epitope not present or possibly not exposed in the native 1,25VDR$_{nuc}$. A recent report has demonstrated that 1,25 D_3-MARRS is, in fact, identical to the multifunctional protein ERp57 [Nemere et al., 2004]. The anti-1,25VDR$_{nuc}$ antibody recognizes a protein of approximately the same molecular weight as 1,25 D_3-MARRS in parathyroid extracts suggesting that the 1,25 D_3-MARRS and 1,25VDR$_{nuc}$ may share some structural features. The parathyroid sub-cellular distribution of the 1,25 D_3-MARRS was also briefly investigated. In chick enterocytes, Nemere et al. [2000] found predominant localization of the 1,25 D_3-MARRS in basolateral membranes, followed by endoplasmic reticulum (ER), which is consistent with the known distribution of ERp57. ERp57 has been well characterized as an endoplasmic reticulum chaperone [Zapun et al., 1998]. We observed roughly equal proportions of 1,25 D_3-MARRS/total protein in crude nuclear, cytosolic, and cell plasma membrane preparations. This method allowed for any unlysed cells and cell debris to be pelleted and included with the nuclear fraction, and cytosolic fractions were also found to contain a significant amount of nuclear-derived protein. However, the cell plasma membrane fraction was found to be free of nuclear-derived protein (histone H1). Therefore 1,25 D_3-MARRS detected in this fraction likely originated in the cell plasma membrane.

As mentioned above, ERp57/1,25 D_3-MARRS has been characterized as an ER chaperone protein with redox function. It has been suggested that ERp57/1,25 D_3-MARRS may provide a link between energy metabolism and ionic regulation [Nemere et al., 2004]. In the ER, ERp57/1,25 D_3-MARRS functions along with calreticulin and calnexin to facilitate proper protein folding [Corbett et al., 1999]. In addition to the ER, ERp57/1,25 D_3-MARRS has been found in the cytosol and membrane microdomains (lipid rafts), and may be involved in STAT signaling [Sehgal, 2003]. The significance of these mechanisms in PHF regulation and vice versa remains to be elucidated.

In this study, we also found that 24,25-$(OH)_2D_3$ has rapid effects on PHF secretion from both SHR and WKY parathyroid cells. Unlike 1,25-$(OH)_2D_3$, the effects of 24,25-$(OH)_2D_3$ on PHF release were inhibitory. Opposing effects of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ have often been described for several tissues where these steroids have been found to exert rapid effects [Khoury et al., 1995; Shan et al., 1996]. It is not known whether the effects of 24,25-$(OH)_2D_3$ on PHF release observed here are receptor-mediated, but a novel membrane receptor for 24,25-$(OH)_2D_3$ (24,25VDR_{mem}) distinct from 1,25 D_3-MARRS [Boyan et al., 2002] has been identified and characterized in chondrocytes [Pedrozo et al., 1999; Boyan et al., 2002], osteoblasts [Boyan et al., 2002], and enterocytes [Nemere et al., 2002]. To our knowledge, a specific antibody to this receptor is not yet available but it will be interesting to examine expression of this putative receptor in the parathyroid of both SHR and WKY rats. It is also possible that 24,25-$(OH)_2D_3$ exerts its effects via a calcitriol receptor or that 24,25-$(OH)_2D_3$ acts through a non-receptor-mediated pathway perhaps by altering membrane composition or signaling via lipid rafts.

The dose-response curves for regulation of PHF secretion by 24,25-$(OH)_2D_3$ exhibited some irregular features. After 30-min exposure, the dose-response curve for WKY, but not SHR, parathyroid cells displayed an unusual non-sigmoidal logarithmic pattern, whereas after 48 h, SHR, but not WKY, parathyroid cells responded in this unusual manner. This may indicate the involvement of multiple receptors such as those mentioned above. These receptors may be differentially expressed between strains and/or may have temporal component to their expression patterns. It is possible that high concentrations of 24,25-$(OH)_2D_3$ can activate 1,25 D_3-MARRS resulting in stimulation of PHF release. Combined inhibitory and stimulatory effects in response to high concentrations of 24,25-$(OH)_2D_3$ could account for the absence of a plateau in this concentration range. Alternatively, 24,25-$(OH)_2D_3$ may modulate autocrine/paracrine regulators of PHF release resulting in a non-classical dose-response curve. A third, but less likely, possibility is that 24,25-$(OH)_2D_3$ is converted to 1,24,25-$(OH)_3D_3$ by parathyroid cytochrome P450 hydroxylases and this metabolite can modulate PHF secretion.

The regulation of PHF release by vitamin D_3 metabolites and the abnormalities in parathyroid sensitivity to these metabolites in the SHR may play a significant role in the etiology of hypertension in this strain, and the findings may possibly extend to low-renin hypertension. When hypertensive patients are classified according to plasma renin activity, the low-renin subset (representing approximately 30–40% of essential hypertension) exhibits, concurrent with elevated PHF, a plasma calcium deficit accompanied by appropriate shifting of the calcium-regulating hormones resulting in increased plasma PTH, elevated 1,25-$(OH)_2D_3$, and suppressed calcitonin [Resnick et al., 1986]. In the Dahl-salt sensitive rat [Kotchen et al., 1990], elevated 1,25-$(OH)_2D_3$ precedes the onset of hypertension. In the SHR, a low-renin model of hypertension, the 1,25-$(OH)_2D_3$ status appears to depend on a variety of factors [Schedl et al., 1988]. Nonetheless, there have been several reports of elevated 1,25-$(OH)_2D_3$ coincident with [Kawashima and Sokabe, 1986] or preceding the development of hypertension [Kawashima and Sokabe, 1986; Lau et al., 1986]; although there appears to be an age-related decline in 1,25-$(OH)_2D_3$ [Bourgouin et al., 1990]. Taken together, these findings suggest that vitamin D_3 metabolites and altered sensitivity to them in the PTG may play a key role in the development of low-renin hypertension through abnormal regulation of PHF release.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Richard Lewanczuk, Dr. Svetlana Krylova, and Dr. Fang Ba for helpful scientific critique and technical assistance, and Ms. Teresa Labedz, Ms. Haiyan Jiao, Michael Lin, and Steven Lin for assistance in isolation of parathyroid tissue and ELISA development.

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