Intestinal Cell Calcium Uptake and the Targeted Knockout of the 1,25D₃-MARRS (Membrane-associated, Rapid Response Steroid-binding) Receptor/PDIA3/Erp57*

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We have crossed ERp57lox/lox mice with commercially available mice expressing villin-driven cre-recombinase. Lysates of intestinal epithelial cells were prepared from knock-out (KO) mice and littermates (LM) and used in Western blot analyses with Ab099 against the N terminus of the 1,25D₃-MARRS (membrane-associated, rapid response steroid-binding) receptor: LM mice exhibited one positive band, which was absent in preparations from KO mice. Saturation analyses of cell lysates with [³H]1,25D₃ revealed negligible binding in preparations from either female or male KOs. Lysates from female and male LM mice had similar affinities but different numbers of binding sites. Isolated enterocytes were tested for steroid-stimulated calcium uptake. Treatment of cells from female or male LM mice with 1,25D₃ elicited enhanced calcium uptake in females and males within 5 min. Intestinal cells from KO mice exhibited a severely blunted or completely absent response to hormone. Confocal microscopy of intestinal cells revealed the presence of cell surface vitamin D receptors. However, antibodies to the vitamin D receptor failed to block 1,25D₃-stimulated calcium uptake. In chick enterocytes we have found that the PKA pathway mediates calcium uptake. The time course for activation of PKA in mouse enterocytes paralleled that for enhanced calcium uptake and for LM females reached 250% of controls within 5 min, and 150% of controls in cells prepared from LM males. Enterocytes from female or male KO mice failed to exhibit steroid hormone-stimulated PKA activity, but did respond to forskolin with enhanced calcium uptake. We conclude that the 1,25D₃-MARRS receptor is of central importance to steroid hormone-stimulated calcium uptake in mammalian intestinal cells.

Experimental Procedures

Animals—Mice with conditional ERp57 deficiency were generated as follows (12). Genomic DNA encoding ERp57 was obtained from a 129/SV genomic DNA library (Resource Center of the German Human Genome Center, Berlin, Germany). Exons 2 and 3 were flanked by two loxP sites, and a neomycin-resistant thymidine kinase gene cassette flanked by two flip-recombinase recognition target sites was inserted between exon 3 and the downstream loxP site. The gene construct was then electroporated into 129P2/OlaHsd mouse embryonic stem cell line 14.1. After neomycin selection, embryonic stem cells were screened by Southern blot analysis to confirm homologous recombination at the 3’ and 5’ flanking regions of the gene construct. A “floxed” embryonic stem cell clone was injected into C57BL/6 blastocysts and was transplanted into pseudopregnant BALB/c mice. Chimeric mice were bred with C57BL/6 mice, and F1 mice were interbred to obtain Pdia3lox/flx offspring. ERp57lox/flx mice (12) were bred to commercially available mice having the cre-recombinase gene driven by the villin promoter (Jackson Laboratories). Pups were weaned at 3 weeks of age and genotyped using the following primers and genomic responses have also been documented for other steroid hormones (8, 9). It is now well accepted that steroid hormones are capable of acting through membrane-localized receptors to initiate pre-genomic effects as well as through regulation of gene expression (9). The identity of these cell surface receptors, however, has been the subject of debate. For the steroid hormone 1,25(OH)₂D₃, two possible candidates exist: the classical vitamin D receptor (VDR) (10) and the more recently discovered and unrelated 1,25D₃-MARRS (membrane-associated, rapid response, steroid binding) receptor/PDIA3/Erp57 (11). Wali et al. (7) have reported that the VDR is not required for the rapid actions of 1,25(OH)₂D₃ in mouse osteoblasts, whereas Mizwicki et al. (10) have argued that only the VDR is necessary to mediate the rapid actions of 1,25(OH)₂D₃, despite our reports that RNAi against the 1,25D₃-MARRS receptor, as well as preincubation of intestinal cells with our neutralizing antibody to the 1,25D₃-MARRS receptor, eliminates the rapid actions of 1,25(OH)₂D₃ on phosphate uptake (11).

In the studies described in this report, genetically engineered mice are used to produce a targeted knock-out of the 1,25D₃-MARRS receptor in intestinal epithelial cells and are tested, along with littermates, for their response to the steroid hormone 1,25(OH)₂D₃.

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3 The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; GBSS, Gey’s balanced salt solution; LM, littermate; MARRS, membrane-associated, rapid response, steroid-binding; VDR, vitamin D receptor.
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classical PCR: ERp57, CGC CAG CCT CTC CAT TTA G (forward) and CAG AGA TCC TGC CTC TG (reverse). The ERp57 product for the littermate (LM) is 100 bp, for the floxed allele the product is 387 bp. For cre-recombinase we used the following primers: GCT GGT TAG CAC CGG AGG TGT AGA G (forward), CGC CAT CTT CCA GCA GGC GCA CC (reverse), to give a 500-bp product. Reaction products were separated out on 2% agarose gels containing ethidium bromide. Mice were fed Harlan Teklad diet 8604 containing 1.36% calcium and 1.01% phosphorus.

Cell Isolation and Incubation Protocols—Mice were used at 8 weeks of age. They were killed by cervical dislocation, and the entire small intestine was removed to ice-cold saline. After 15 min, the intestines were slit longitudinally, rinsed in ice-cold saline, and transferred to citrate chelation medium (96 mM NaCl, 27 mM citric acid, 1.5 mM KCl, 5.6 mM NaHPO₄, 8 mM KH₂PO₄, pH 5.0). The acidic pH allows retention of viability and morphology in chick intestinal cells (13) and, as shown below, allows culturing of mouse intestinal cells as well. The intestines were stirred for 15 min at room temperature to dissociate epithelial cells and then transferred to fresh chelation medium. Microscopic observation confirmed the presence of differentiated and crypt cells. The released cells were poured into 50-ml conical centrifuge tubes and held on ice. Two additional 15-min periods of cell isolation were conducted, and the cells were pooled and collected by centrifugation at 1000 × g, 10 min (4 °C). The cell pellets were resuspended in 10 ml of Gey’s balanced salt solution (GBSS; containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH₂PO₄, 0.84 mM NaHPO₄, 1.03 mM MgCl₂6H₂O, 0.28 mM MgSO₄7H₂O, 0.9 mM CaCl₂, pH 7.3). Isolated intestinal epithelial cells from male and female mice were kept separate and not pooled. For calcium uptake studies, aliquots of cell suspension (2.2 ml) were added to 50-ml conical centrifuge tubes containing 1 µCi/ml ⁴⁵CaCl₂ (PerkinElmer Life Sciences), at t = -10 min. Samples (100 µl) were removed at t = -5 and -1 min to establish basal uptake, and test substances or vehicle controls were added at t = 0 min. Additional samples were removed at 1, 3, 5, 7, and 10 min during the treated phase. All aliquots were pipetted into 1000 µl of ice-cold GBSS to stop uptake and dilute radionuclide, then centrifuged at 1000 × g, 10 min (4 °C). After decanting the supernatants, the inside of the tubes, while still inverted, were swabbed with a tissue to remove residual supernatant. Cell pellets were resuspended in 500 µl of reagent-grade water and analyzed for radioactivity and protein using the Bradford reagent (Bio-Rad). After blocking nonspecific binding sites (Upstate) using [³²P]ATP and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrates as previously described (15).

Western Blot Analysis—Isolated intestinal epithelial cells were resuspended in reagent-grade water, protein was determined; they were combined with SDS-PAGE sample buffer and heated at 95 °C for 5 min. Samples were separated on 8% SDS-PAGE and electroblotted onto a PVDF membrane using a semidry blotting apparatus (Bio-Rad). After blocking nonspecific binding sites (15), membranes were incubated with Ab099 against the N terminus of the 1,25D₃-MARRS receptor at a concentration of 1:5000. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG.

FIGURE 1. Intestinal cells from LM mice have a single immunoreactive band identifying the 1,25D₃-MARRS receptor, which is lacking in intestinal cells from targeted KO mice. Intestinal cells were isolated by citrate chelation and homogenized. Samples were resolved on 8% SDS-PAGE and blotted onto a PVDF membrane. Western blot analysis was performed on authentic chick basal lateral membranes (lanes 1 and 6), 15 µg of intestinal cell lysate protein from LM and KO mice (lanes 2 and 3, respectively), or 30 µg of protein from LM and KO mice (lanes 4 and 5, respectively). Ab099 against the chick 1,25D₃-MARRS receptor was used at a concentration of 1:5000. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG.

Saturation Analyses for [⁳H]1,25(OH)₂D₃ Binding—Because ERp57 has been found to be located intracellularly as well as on the cell surface, whole cell lysates were used to confirm the presence or absence of binding. Isolated intestinal epithelial cells were homogenized in 10 mM Tris, 1.5 mM EDTA, 2 mM dithiothreitol (TED), pH 7.4, protein was determined, and 50 (0.01%, final concentration) or 300 pM 1,25(OH)₂D₃ at t = 0 min. Additional samples were removed at t = 1, 3, 5, 7, and 10 min, and the cells were collected by centrifugation. Cell lysates were analyzed for enzyme activity according to kit instructions (Upstate) using [³²P]ATP and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrates as previously described (15).
μg was added to each of six tubes. Three tubes were used for determination of total binding and three for determination of nonspecific binding. The tubes for total binding received increasing concentrations of [3H]1,25(OH)2D3, whereas tubes for determining nonspecific binding received the same concentration of radiolabeled metabolite and a 200-fold molar excess of unlabeled 1,25(OH)2D3. After a 5-h incubation on ice, bound and free hormone were separated by the perchloric acid precipitation procedure. Values represent mean ± range for two independent experiments.

**Statistics and Data Analysis**—The specific binding of [3H]1,25(OH)2D3 to the 1,25D3-MARRS receptor was calculated and plotted against the corresponding concentration of [3H]1,25(OH)2D3. The data were analyzed by nonlinear regression analysis by fitting to either a three-parameter sigmoid equation (which would demonstrate positive cooperativity) or a hyperbolic function (noncooperative interaction) as previously described (15). The sigmoid equation was as follows,

\[
Y = \frac{a}{1 + e^{-(x - xo)/b}} \quad \text{(Eq. 1)}
\]

where \( Y \) is specifically bound [3H]1,25(OH)2D3 (femtomoles/milligram of protein), \( x \) is the nanomolar concentration of 1,25(OH)2D3 in the incubation mixture, \( a \) is the maximum specifically bound [3H]1,25(OH)2D3, \( xo \) is the concentration of 1,25(OH)2D3 in the incubation mixture at half-maximum specifically bound [3H]1,25(OH)2D3 (\( kD \)), and \( b \) is the minimum specifically bound [3H]1,25(OH)2D3. For the hyperbolic function, the equation was as follows,

\[
Y = \frac{a*x}{B + x} \quad \text{(Eq. 2)}
\]

where \( Y \) is specifically bound [3H]1,25(OH)2D3 (femtomoles/milligram of protein), \( x \) is the nanomolar concentration of 1,25(OH)2D3 in the incubation mixture, and \( a \) is the maximum specifically bound [3H]1,25(OH)2D3 (\( B_{max} \)).

**Confocal Microscopy**—Isolated intestinal epithelial cells were cultured in RPMI 1640 medium lacking fetal bovine serum overnight in 35-mm plastic Petri dishes containing a glass coverslip. The following morning, media were aspirated and the cells fixed in 4% paraformaldehyde, 3% sucrose in phosphate-buffered saline (PBS) for 30 min. After three washes with PBS, the coverslips were overlaid with Texas Red-conjugated secondary antibody and incubated for an additional 30

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**FIGURE 2.** Saturation analyses of [3H]1,25(OH)2D3 binding to intestinal cell lysates of female and male mice. Intestinal cells were isolated by citrate chelation and incubated in triplicate with the indicated concentrations of radiolabeled hormone without (total binding) or with a 200-fold molar excess of unlabeled steroid hormone (nonspecific binding). Bound and free hormone were separated by the perchloric acid precipitation procedure. Values represent mean ± S.E. for two independent experiments.

**FIGURE 3.** Intestinal cells from male LM mice respond to 1,25(OH)2D3 with enhanced calcium uptake, whereas intestinal cells from KO mice do not. Cells were isolated by citrate chelation and resuspended in GBSS. At \( t = 10 \) min, cell suspensions were added to tubes containing 45CaCl2. Baseline samples were taken at \( t = -5 \) and \( -1 \) min, and 300 pm 1,25(OH)2D3 (final concentration) or the vehicle ethanol (0.01%, final concentration) was added. Additional samples were removed at the indicated times. All samples were pipetted into ice-cold GBSS, and cells were collected by centrifugation for analysis of protein and radioactivity. Values represent mean ± S.E. for \( n = 6 \) independent experiments. *, \( p < 0.05 \) relative to corresponding controls.
min. After a final three washes, the coverslips were mounted on microscope slides. Primary antibody was omitted for negative staining controls. A Bio-Rad MRC 1024 laser-scanning confocal microscope system mounted in the Keller position and attached to a Nikon TE-200 microscope that was used for confocal imaging. The krypton-argon laser produced three excitation lines of 488 nm, 568 nm, and 647 nm. The emission filter consisted of a 522/32 bandpass filter that collected all light between 506 and 538 nm. Images were collected with Bio-Rad LASERSHARP acquisition software, using a 40× objective.

RESULTS

Western Blotting and Saturation Analyses—Fig. 1 depicts the results of Western blot analysis of intestinal cell lysates of LM and targeted knock-out (KO) mice. Lanes 1 and 6 contained purified chick basal lateral membranes as positive controls. Intestinal epithelial cells from littermate mice contained a single immunoreactive band at 15 and 30 μg of protein, whereas an equivalent band was missing in KO mice (Fig. 1). Isolated intestinal epithelial cell lysates were then tested for specific [3H]1,25(OH)2D3 binding in saturation analyses. The adjusted coefficients of variation for sigmoidal fits were 0.848 and 0.849 for LM females and males, respectively. Equivalent adjusted coefficients of variation for hyperbolic fits were lower. KO mice of either sex exhibited very little specific binding (Fig. 2).

Calcium Uptake in Male Mice—Intestinal epithelial cells isolated from male LM mice responded to 300 pM 1,25(OH)2D3 within 1 min with a significant increase in 45Ca uptake, relative to corresponding controls, reaching 150% of corresponding controls within 5 min of exposure to hormone (Fig. 3). In comparison, enterocytes isolated from male KO mice failed to respond to 1,25(OH)2D3 with enhanced calcium uptake, relative to controls (Fig. 3). Although the decrease in calcium uptake in enterocytes isolated from LM mice might appear to be dependent on the 1,25D3-MARRS receptor, the observation that forskolin eliminates it (see below) would argue against this.

We have previously reported (17) that in chick intestinal cells the PKA pathway mediates calcium uptake. We therefore tested the effect of 20 μM forskolin on calcium uptake in intestinal epithelial cells isolated from mice. Fig. 4 illustrates the results of experiments in which enterocytes from either LM or KO mice were treated with the activator of adenylate cyclase. Both LM and KO mice responded to forskolin with enhanced 45Ca uptake relative to corresponding controls, demonstrating that the targeted deletion of the 1,25D3-MARRS receptor did not seriously alter the signaling pathway. In contrast, direct activation of the PKC signaling pathway with phorbol ester failed to enhance calcium uptake in either LM or KO mice (Fig. 5).

Calcium Uptake in Female Mice—Intestinal epithelial cells isolated from female LM mice responded to 300 pM 1,25(OH)2D3 with a significant increase in calcium uptake within 1 min, reaching 175% of corresponding controls by 5 min of incubation with steroid (Fig. 6). Enterocytes isolated...
from female KO mice exhibited a blunted response to the seco-steroid that was significantly different from corresponding controls at 1, 3, and 10 min after hormone (Fig. 6). When forskolin was used in incubations with enterocytes from female LM and KO mice, stimulated calcium uptake was observed (Fig. 7), although the uptake curves did not closely parallel those seen with 1,25(OH)_{2}D_{3} (Fig. 5). Likewise, incubation of enterocytes from LM and KO female mice with phorbol ester resulted in a modest enhancement of calcium uptake, relative to corresponding controls (Fig. 8). In contrast, incubation of isolated intestinal epithelial cells from LM female mice with both forskolin and phorbol ester resulted in an enhanced calcium uptake to 180% of controls (Fig. 9), an increase that parallels that seen with 1,25(OH)_{2}D_{3}.

**Protein Kinase A Activity**—Intestinal cells isolated from female LM and KO mice were incubated with 300 pM 1,25(OH)_{2}D_{3} or vehicle in time course studies to determine PKA activation. As shown in Fig. 10, cells from LM mice exhibited significantly greater PKA activity 1–5 min after steroid, relative to corresponding controls, achieving a maximum of 250% of controls at 5 min and thereafter declining toward baseline levels. Parallel experiments with cells from KO mice revealed an absence of response to steroid (Fig. 10). Likewise, enterocytes from LM male mice responded to 300 pM 1,25(OH)_{2}D_{3} with significant increases in PKA activity, reaching a maximum of 250% at 7 min and remaining elevated at 10 min (Fig. 11). Isolated intestinal cells from male KO mice did not exhibit enhanced PKA activity in response to steroid and indeed, showed inhibition after steroid at 1 and 10 min after 1,25(OH)_{2}D_{3} (Fig. 11).

**Confocal Microscopy**—Because all of the responses examined to this point were mediated by the 1,25D_{3}-MARRS receptor, isolated intestinal epithelial cells were examined for the presence of cell surface VDR by confocal microscopy. As shown in Fig. 12, cells from either LM or KO mice exhibited patches of cell surface VDR immunoreactivity.

**Lack of Cell Surface VDR Involvement in Calcium Uptake**—Finally, enterocytes from LM mice were preincubated with either polyclonal or monoclonal antisera to the VDR and then tested for calcium uptake in response to 300 pM 1,25(OH)_{2}D_{3}. Because the results for either antibody were similar, the data were combined. As shown in Fig. 13, Antibodies against the VDR failed to inhibit the 1,25(OH)_{2}D_{3}-mediated increase in calcium uptake by isolated intestinal epithelial cells from either LM female or male mice.
DISCUSSION

The present work demonstrates that the targeted disruption of the 1,25D₃-MARRS receptor gene in intestinal epithelial cells eliminates the rapid response to 1,25(OH)₂D₃ with respect to rapidly enhanced calcium uptake and PKA signaling. Such pre-genomic effects of the steroid hormone are likely important physiologically because transit of calcium through the duodenum requires only minutes. During the course of this work, a number of sex-related differences were observed. Although saturation analyses of intestinal cell lysates revealed that in male and female mice the 1,25D₃-MARRS receptors have similar affinities for the steroid hormone, the Bₘₐₓ for the receptor in

FIGURE 8. Intestinal cells from female LM and KO mice responded to phorbol ester with enhanced calcium uptake. Experimental conditions were as described in the legend to Fig. 3, except that cells were treated either with 100 nm phorbol myristate acetate (PMA) or the vehicle ethanol (0.04%, final concentration), and cells were collected by centrifugation for analysis of protein and radioactivity. Values represent mean ± S.E. for n = 6 independent experiments. *, p < 0.05 relative to corresponding controls.

FIGURE 9. Addition of both forskolin and phorbol ester to intestinal cells of female LM mice resulted in enhanced calcium uptake that paralleled the response observed with 1,25(OH)₂D₃. Values represent mean ± S.E. for n = 4 independent experiments. *, p < 0.05 relative to corresponding controls.

FIGURE 10. Intestinal cells from female LM mice responded to hormone with enhanced PKA activity, whereas cells from KO mice did not. Experimental procedures were as described in Fig. 3 without the addition of radioactivity. Cells were lysed, and 10 μg of protein was assayed for PKA activity with Kemptide and [³²P]ATP as substrate. Values represent mean ± S.E. for n = 4 independent experiments. *, p < 0.05 relative to corresponding controls.

FIGURE 11. Intestinal cells from male LM mice responded to hormone with enhanced PKA activity, whereas cells from KO mice did not. Experimental procedures were as described in Fig. 3 without the addition of radioactivity. Cells were lysed, and 10 μg of protein was assayed for PKA activity with Kemptide and [³²P]ATP as substrate. Values represent mean ± S.E. for n = 3 independent experiments. *, p < 0.05 relative to corresponding controls.
female mice was approximately half of that observed in male mice. In male mice, the PKA pathway mediates calcium uptake, which is what we have previously reported for hormone-stimulated calcium uptake in male chicks (17). However, in enterocytes from female mice, both the PKA and PKC pathways mediate calcium uptake. Equivalent experiments have not been performed with enterocytes from hens, so it is difficult to say whether this represents a sex-specific or species-specific difference. In addition, 1,25(OH)2D3 stimulated PKA activation in intestinal epithelial cells from male and female mice to a similar extent, but the time courses were different with respect to onset of stimulation.

Finally, we determined by confocal microscopy that intestinal cells from both LM and KO mice contain similar staining patterns for cell surface VDR; however, antibodies against the VDR failed to inhibit 1,25(OH)2D3-mediated stimulation of calcium uptake. We have previously reported that chick intestinal cells rely solely on the 1,25D3-MARRS receptor to mediate steroid hormone effects on enhanced calcium uptake (14, 17), whereas 1,25(OH)2D3-stimulated calcium uptake in isolated rat enterocytes is inhibited by either antibodies to the 1,25D3-MARRS receptor or the VDR (14). This has led us to postulate that the evolution of mammals may have led to the dual cell surface receptor mechanism. In view of the present findings with mice enterocytes, this appears not to be the case. It remains to be determined what the function of cell surface VDR is in intestinal cells of mice. One possibility might be to mediate the actions of 25-hydroxyvitamin D3, a metabolite that has been shown to increase calcium uptake in chick intestinal cells at physiological concentrations (18) and which has been reported to bind to the VDR in prostate cancer cell lines (19). The 1,25D3-MARRS receptor has protein disulfide isomerase activity through two thioredoxin domains (11) and was
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originally identified as the chaperone ERp57. Primm and Gilbert (20) have previously reported that the parental protein, PDI is capable of binding estradiol and thyroid hormone. Thus, it may be that this family of proteins can act as alternate receptors for multiple steroid hormones.

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