J Am Soc Nephrol 13: 2102–2109, 2002

1,25-Dihydroxyvitamin D_3-Independent Stimulatory Effect of Estrogen on the Expression of ECaC1 in the Kidney

MONIQUE VAN ABEL,* JOOST G. J. HOENDEROP,* OLIVIER DARDENNE,† RENÉ ST. ARNAUD,† CAREL H. VAN OS,* HANS J. P. T. M. VAN LEEUWEN,‡ and RENÉ J. M. BINDELS*

**Department of Cell Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, Nijmegen, The Netherlands; †Genetics Unit, Shriners Hospital for Children, Montreal, Quebec, Canada; and ‡Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.*

Abstract. Estrogen deficiency results in a negative Ca^{2+} balance and bone loss in postmenopausal women. In addition to bone, the intestine and kidney are potential sites for estrogen action and are involved in Ca^{2+} handling and regulation. The epithelial Ca^{2+} channel ECaC1 (or TRPV5) is the entry channel involved in active Ca^{2+} transport. Ca^{2+} entry is followed by cytosolic diffusion, facilitated by calbindin-D_{28K} and/or calbindin-D_{9k}, and active extrusion across the basolateral membrane by the Na^+/Ca^{2+}-exchanger (NCX1) and plasma membrane Ca^{2+}-ATPase (PMCA1b). In this transcellular Ca^{2+} transport, ECaC1 probably represents the final regulatory target for hormonal control. The aim of this study was to determine whether 17β-estradiol (17β-E_2) is involved in Ca^{2+} reabsorption via regulation of the expression of ECaC1. The ovariectomized rat model was used to investigate the regulation of ECaC1, at the mRNA and protein levels, by 17β-E_2 replacement therapy. Using real-time quantitative PCR and immunohistochemical analyses, this study demonstrated that 17β-E_2 treatment at pharmacologic doses increased renal mRNA levels of ECaC1, calbindin-D_{28K}, NCX1, and PMCA1b and increased the protein abundance of ECaC1. Furthermore, the involvement of 1,25-dihydroxyvitamin D_3 in the effects of 17β-E_2 was examined in 25-hydroxyvitamin D_3-1α-hydroxylase-knockout mice. Renal mRNA expression of calbindin-D_{9K}, calbindin-D_{28K}, NCX1, and PMCA1b was not significantly altered after 17β-E_2 treatment. In contrast, ECaC1 mRNA and protein levels were both significantly upregulated. Moreover, 17β-E_2 treatment partially restored serum Ca^{2+} levels, from 1.63 $\pm$ 0.06 to 2.03 $\pm$ 0.12 mM. In conclusion, this study suggests that 17β-E_2 is positively involved in renal Ca^{2+} reabsorption via the upregulation of ECaC1, an effect independent of 1,25-dihydroxyvitamin D_3.

Ca^{2+} homeostasis plays a key role in mammalian development and function and is maintained by the function of the small intestine, skeleton, and kidney. The classic hormones involved in Ca^{2+} homeostasis include 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], parathyroid hormone, and calcitonin (1,2). Estrogen is usually not considered a calciotropic hormone, but the idea that estrogen plays a role in Ca^{2+} homeostasis has been widely accepted (3). This involvement is clearly illustrated by the fact that estrogen deficiency results in a negative Ca^{2+} balance and bone loss among postmenopausal women (4). In addition to bone, two potential sites of estrogen action are the intestine and kidney, which are involved in Ca^{2+} handling and regulation.

There is increasing evidence that estrogen plays a physiologic role in the regulation of renal Ca^{2+} reabsorption. *In vivo* studies demonstrated that estrogen deficiency was associated with increased renal Ca^{2+} loss, which could be corrected with estrogen replacement therapy (5,6). Furthermore, there is evidence that estrogen receptors (ER) are also present in the kidney (7,8). ER localization studies have suggested both proximal and distal tubules as possible sites of action (9,10). However, the underlying mechanism by which estrogen might affect renal Ca^{2+} handling is still poorly understood. In addition, there is considerable disagreement regarding possible direct effects of estrogen on Ca^{2+} reabsorption *versus* indirect effects produced via actions on vitamin D metabolism.

The epithelial Ca^{2+} channel ECaC1 (or TRPV5), which is observed in renal distal convoluted tubule and connecting tubule cells, is the entry channel involved in transcellular Ca^{2+} transport (11). Ca^{2+} entry via ECaC1 is followed by cytosolic diffusion, facilitated by Ca^{2+}-binding proteins (calbindin-D_{28K} and/or calbindin-D_{9k}), and active extrusion of Ca^{2+} across the basolateral membrane by a high-affinity plasma membrane Ca^{2+}-ATPase (PMCA1b) and a Na^+/Ca^{2+}-exchanger (NCX1). In this active process, ECaC1 probably forms the final regulatory target for hormonal control (12,13). Therefore, ECaC1 could be a target for estrogen in the regulation of Ca^{2+} reabsorption.

The aim of this study was to determine whether estrogen acts on the distal part of the renal tubule by increasing the expression of ECaC1, which could result in increased Ca^{2+} reabsorp-

Received April 3, 2002. Accepted April 29, 2002.
Correspondence to Dr. René J. M. Bindels, 160 Cell Physiology, University Medical Center Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands. Phone: +31-24-3614211; Fax: +31-24-3616413; E-mail: r.bindels@ncmls.kun.nl

1046-6673/1308-2102
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000022423.34922.2A

tion. We used the ovariectomized (OVX) rat model of estrogen deficiency to investigate changes in ECaC1, at the mRNA and protein levels, with estrogen replacement therapy. The involvement of the important calciotropic hormone $1,25(OH)_2D_3$ in estrogen effects was examined in 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase)-knockout mice, as a vitamin D-deficient model (14). This study indicates that estrogen upregulates ECaC1 expression in the kidney, thereby regulating Ca^{2+} reabsorption, and that estrogen exerts this effect independently of $1,25(OH)_2D_3$.

Materials and Methods

Animals

Experiment 1. Twenty-five mature, virgin, female, Wistar rats (Hsd/Cpd:Wu, bred specific pathogen-free by Harlan, CPB, Zeist, The Netherlands), weighing 225 to 250 g, were housed individually in Macrolon cages, in a light- and temperature-controlled room (14-h light/10-h near-dark cycle, at 21 to 23°C). The rats received 16 g of standard pelleted food daily, and water was available *ad libitum*. On day 1 of the experiment, the rats were weighed and divided into five groups. After anesthesia induction, bilateral OVX or a sham operation was performed. Thereafter, rats received 17β-estradiol (17β-E_2) (Sigma Chemical Co., St. Louis, MO) or vehicle (gelatin and mannitol), added to the pelleted food, each day. Sham-operated animals served as control animals. OVX animals were given either the vehicle alone, 2 $\times$ 32 μg 17β-E_2/d, 2 $\times$ 125 μg 17β-E_2/d, or 2 $\times$ 500 μg 17β-E_2/d. Treatment was started immediately after OVX and lasted for 7 d. Before the end of treatment, overnight urine samples were collected. On the last day, the rats were euthanized. Blood was collected from the abdominal aorta, the uterus was excised and weighed, and the kidneys were dissected and immediately frozen in liquid nitrogen.

Experiment 2. Homozygous, 9-wk-old, male, 1α-OHase-knockout mice (14) were housed individually in a light- and temperature-controlled room. Both deionized water and standard pelleted food were available *ad libitum* throughout the experiment. The mice were randomized into the following two groups (with each group consisting of four mice): homozygous mice and homozygous mice given infusions of 17β-E_2. Osmotic minipumps (model 1007D; Alzet, DURECT Corp., Cupertino, CA) were used for rate-controlled delivery of 17β-E_2; these pumps release their contents at a rate of 0.5 μl/h for 7 d. The pumps were filled with the desired solution and implanted subcutaneously in the backs of the mice. The infusion dose for the 17β-E_2-treated mice was 10 μg/d, and control mice received vehicle solution alone (15% ethanol/50% DMSO/35% water, vol/vol). After 7 d, mice were euthanized, blood was collected via orbital puncture, and kidneys were dissected and immediately frozen in liquid nitrogen. The animal ethics boards of the University of Nijmegen and Shriners Hospital for Children (Montreal, Quebec, Canada) approved all animal experimental procedures.

Analytical Procedures

Serum Ca^{2+} concentrations were analyzed by using a colorimetric assay kit, as described previously (15). Serum 17β-E_2 levels were measured by using an extraction procedure with diethyl ether, followed by RIA (DPC, Los Angeles, CA) (16).

RNA Isolation, Reverse Transcription, and Quantitative PCR

Total RNA from kidney cortex was isolated by using TriZol reagent (Life Technologies, Breda, The Netherlands), according to the protocol described by the manufacturer. RNA was treated with DNase, to prevent genomic DNA contamination, and was finally resuspended in diethylpyrocarbonate-treated water filtered with a MilliQ system (Millipore, Bedford, MA). Total RNA (2 μg) was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Life Technologies), as described previously (17). Expression levels of renal ECaC1, calbindin-D_{28K}, calbindin-D_{9K}, NCX1, and PMCA1b mRNA were quantified by real-time quantitative PCR, using an ABI Prism 7700 sequence detection system (PE Biosystems, Rotkreuz, Switzerland). The expression level of hypoxanthine-guanine phosphoribosyl transferase was used as an internal control, to normalize differences in RNA extractions, the degree of RNA degradation, and reverse transcription efficiencies. Primers and probes targeting the genes of interest were designed by using Primer Express software (Applied Biosystems, Foster City, CA) and are listed in Table 1. The 3'-ends of the probes were labeled with the quencher dye 6-carboxytetramethylrhodamine. The 5'-ends of all probes were labeled with the reporter dye 6-carboxyfluorescein (Biolegio, Malden, The Netherlands).

Immunohistochemical Analyses

Seven-micron sections of frozen kidney tissue were cut with a cryotome and collected on SuperFrost/Plus-coated (Menzel-Glazer, Germany) glass slides. For ECaC1 staining, sections were incubated in boiled citrate buffer (0.01 M sodium citrate, 0.01 M citric acid, pH 6), cooled for 30 min, and washed three times with TN buffer (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5). For immunoperoxidase staining, sections were incubated for 30 min at room temperature in TN buffer containing 0.3% (vol/vol) H_2O_2 and were washed three times with TN buffer. Sections were then incubated for 30 min in TNB buffer (TN buffer containing 0.5%, wt/vol, blocking reagent; NEN Life Science Products, Perkin Elmer, Boston, MA). Subsequently, sections were incubated for 16 h at 4°C in TNB buffer containing affinity-purified guinea pig anti-ECaC1 antiserum (1:200) (11). Sections were washed three times with TNT buffer (TN buffer containing 0.05%, vol/vol, Tween 20) and then incubated for 1 h at room temperature with biotin-labeled, affinity-purified, goat anti-guinea pig IgG (1:2000; Sigma Chemical Co., St. Louis, MO). After washing with TNT buffer, sections were incubated with streptavidin-horseradish peroxidase (1:100; NEN Life Science Products) for 30 min at room temperature. Sections were washed three times with TNT buffer and incubated for 7 min with fluorescein tyramide in amplification diluent (1:50; NEN Life Science Products). Sections were then washed, dehydrated in 50 to 100% (vol/vol) methanol, and mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 2.5% (wt/vol) NaN_3. Photographs were taken with a Bio-Rad MRC 1000 confocal laser scanning microscope. The anti-ECaC1 antibody used in this study has been extensively characterized but is, unfortunately, not able to be used for immunoblotting (17). For semiquantitative assessment of ECaC1 protein expression, the relative amounts of immunopositive tubules in 10 to 15 randomly selected microscopic fields were determined for each animal, using a Zeiss Axioskop microscope (Zeiss, Thornwood, NY) equipped for epifluorescence illumination.

Statistical Analyses

Values are expressed as mean $\pm$ SEM. Differences between groups were tested by using one-way ANOVA and were further evaluated by using Fisher's multiple-comparison procedure (18). Differences in means with $P < 0.05$ were considered statistically significant. All analyses were performed by using the Statview statistical package

Table 1. Sequences of primers and Taqman probes for real-time quantitative PCR[a]

Gene		Forward Primer	Reverse Primer	Probe
HPRT	R	5′-TATCAGACTGAAGAGCTACTGTAATGACC-3′	5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′	5′-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3′
	M	5′-TTATCAGACTGAAGAGCTACTGTAATGATC-3′	5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′	5′-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3′
ECaC1	R	5′-CTTACGGGTTGAACACCACCA-3′	5′-TTGCAGAACCACAGAGGCTCTA-3′	5′-TGCTTCTCAGATAGTTGTTCTTGTACTTCCTCCTTGT-3′
	M	5′-CGTTGGTTCTTACGGGTTGAAC-3′	5′-GTTTGGAGAACCACAGAGGCTCTA-3′	5′-TGTTTCTCAGATAGCTGCTCTTGTACTTCCTCTTTGT-3′
CaBP-D28k	R	5′-GGAAGCTGGAGCTGACAGAGAT-3′	5′-TGAACTCTTTCCCACACATTTTGAT-3′	5′-ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA-3′
	M	5′-AACTGACAGAGATGGCCAGGTTA-3′	5′-TGAACTCTTTCCCACACATTTTGAT-3′	5′-ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA-3′
CaBP-D9k	M	5′-CCTGCAGAAATGAAGAGCATTTTT-3′	5′-CTCCATCGCCATTCTTATCCA-3′	5′-CAAAAATATGCAGCCAAGGAAGGCGA-3′
PMCA1b	R	5′-CGCCATCTTCTGCACAATT-3′	5′-CAGCCATTGTTCTATTGAAAGTTC-3′	5′-CAGCTGAAAGGCTTCCCGCCAAA-3′
	M	5′-CGCCATCTTCTGCACCATT-3′	5′-CAGCCATTGCTCTATTGAAAGTTC-3′	5′-CAGCTGAAAGGCTTCCCGCCAAA-3′
NCX1	R	5′-TCCCTATAAAACCATTGAAGCACA-3′	5′-TTTCTCATACTCCTCGTCATCGATT-3′	5′-ACCTTGACTGATATTGTTTTGACTATTTCATCATTCTGGA-3′
	M	5′-TCCCTACAAAACTATTGAAGGCACA-3′	5′-TTTCTCATACTCCTCGTCATCGATT-3′	5′-ACCTTGACTGATATTGTTTTGACTATTTCATCATTCTGGA-3′

[a] PCR primers and fluorescent probes (5′-6-carboxyfluorescein- and 3′-6-carboxytetramethylrhodamine-labeled) were designed by using the computer program Primer Express (Applied Biosystems) and were purchased from Biolegio. HPRT, hypoxanthine-guanine phosphoribosyl transferase; ECaC1, epithelial Ca^{2+} channel 1; CaBP, calbindin; PMCA1b, plasma membrane Ca^{2+}-ATPase; NCX1, Na^+/Ca^{2+}-exchanger; R, rat; M, mouse.

(Power PC version 4.51; Statview, Berkeley, CA) on a Macintosh computer.

Results

Effects of OVX and 17β-E_2 Treatment on Rats

Age-matched, untreated, OVX rats weighed significantly more than rats in the sham-operated group (Table 2). In contrast, 17β-E_2 treatment induced significant reductions in weight, with the greatest decrease being observed for the OVX/high-dose 17β-E_2-treated rats (Table 2). OVX resulted in the expected atrophy of the uterus, which was prevented by 17β-E_2 therapy in OVX/high-dose 17β-E_2-treated rats (Table 2). Serum 17β-E_2 levels were reduced in untreated OVX rats, confirming OVX, whereas supplementation with the highest 17β-E_2 dose resulted in significantly higher serum 17β-E_2 levels (Table 2). 17β-E_2 treatment reduced serum Ca^{2+} levels, resulting in slightly but significantly lower serum Ca^{2+} levels in the OVX/high-dose 17β-E_2-treated group.

Effect of 17β-E_2 on ECaC1 Expression in Rat Kidneys

To investigate the effect of 17β-E_2 on ECaC1 mRNA expression, real-time quantitative PCR was performed with total RNA isolated from kidney cortex (Figure 1A). The data demonstrated that 17β-E_2 supplementation, using the highest dosage, for estrogen-depleted rats resulted in a 2.5-fold increase in ECaC1 mRNA expression, compared with OVX rats. The expression levels of other Ca^{2+}-transport proteins involved in transcellular Ca^{2+} transport, namely calbindin-D_{28K}, NCX1, and PMCA1b, were also determined. More than twofold upregulation of calbindin-D_{28K} mRNA expression was observed in OVX/high-dose 17β-E_2-treated rats, compared with OVX rats (Figure 1B). In addition, significant increases in the expression of both NCX1 (5.5-fold) and PMCA1b (1.5-fold) mRNA in OVX/medium-dose 17β-E_2-treated rats, compared with OVX rats, were demonstrated (Figure 1, C and D). Next, the abundance of ECaC1 protein in kidneys was examined. Figure 2A presents representative immunofluorescence labeling of distal tubules in sections of kidney cortex from OVX and OVX/high-dose 17β-E_2-treated rats. More ECaC1 protein was detected in tissue from the supplemented rats, as indicated by the increased staining in the kidney cortex. In these immunopositive tubules, ECaC1 was localized to the apical membrane of distal tubular segments. For semiquantitative assessment of ECaC1 protein expression, the relative amounts of immunopositive tubules in 10 to 15 randomly selected microscopic fields were counted for each kidney cortex section. Figure 2B presents the average values for each experimental group. The levels of ECaC1 expression in OVX/medium-dose 17β-E_2-treated and OVX/high-dose 17β-E_2-treated rats were significantly higher than those in OVX rats.

17β-E_2-Induced Increases in Plasma Ca^{2+} Levels in 1,25(OH)$_2$D$_3$-Deficient Mice

One of the characteristics of the homozygous 1α-OHase-knockout mice is growth retardation, as illustrated by their low

Table 2. Effects of OVX and 17β-E_2 supplementation in rats[a]

	Sham ($n = 5$)	OVX ($n = 5$)	OVX + E_2L ($n = 5$)	OVX + E_2M ($n = 5$)	OVX + E_2H ($n = 5$)
Body weight (g)					
beginning	207 ± 4	205 ± 4	207 ± 3	209 ± 4	204 ± 2
final	203 ± 4	215 ± 3[b]	207 ± 4	202 ± 4[c]	189 ± 4[d]
Uterus weight (g)	0.51 ± 0.07	0.23 ± 0.04[b]	0.21 ± 0.01	0.39 ± 0.04	0.66 ± 0.13[e]
Serum level					
17β-E_2 (pg/ml)	11 ± 6	5 ± 3	5 ± 1	12 ± 2	64 ± 6[f]
Ca^{2+} (mM)	2.38 ± 0.02	2.40 ± 0.03	2.39 ± 0.02	2.36 ± 0.04	2.31 ± 0.02[c]

[a] OVX, ovariectomized; 17β-E_2, 17β-estradiol; +E_2L, supplemented with 2 × 32 μg 17β-E_2/d; +E_2M, supplemented with 2 × 125 μg 17β-E_2/d; +E_2H, supplemented with 2 × 500 μg 17β-E_2/d. Data are presented as mean ± SEM.
[b] $P < 0.05$ *versus* sham.
[c] $P < 0.05$ *versus* OVX.
[d] $P < 0.05$ *versus* all groups.
[e] $P < 0.05$ *versus* OVX, OVX plus low-dose 17β-E_2, and OVX plus medium-dose 17β-E_2.
[f] $P < 0.0001$ *versus* all groups.

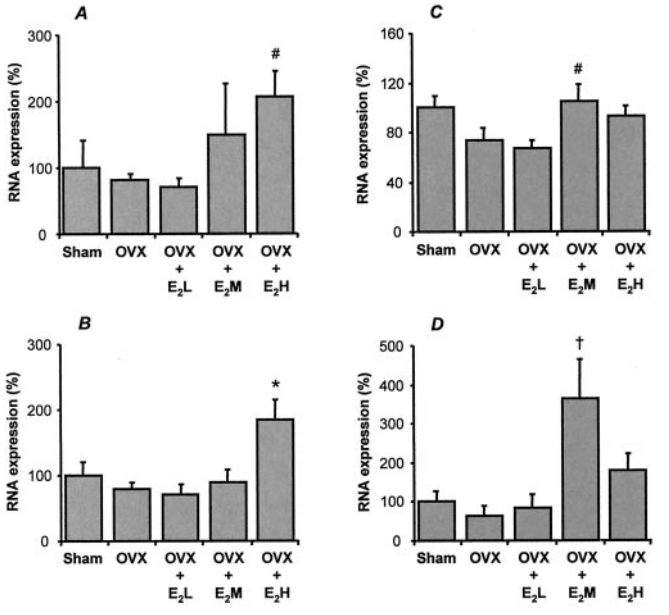

Figure 1. Effects of ovariectomy (OVX) and 17β-estradiol (17β-E_2) supplementation on the mRNA expression levels of ECaC1 and other Ca^{2+}-transport proteins in rat kidneys. mRNA expression levels were measured by using real-time quantitative PCR, as described in Materials and Methods. The expression levels of ECaC1 (A), calbindin-D_{28K} (B), plasma membrane Ca^{2+}-ATPase (PMCA1b) (C), and Na^+/Ca^{2+}-exchanger (NCX1) (D) for the different experimental groups are presented relative to levels in sham-treated rats. +E_2L, supplemented with 2 × 32 μg 17β-E_2/d; +E_2M, supplemented with 2 × 125 μg 17β-E_2/d; +E_2H, supplemented with 2 × 500 μg 17β-E_2/d. Data are presented as means ± SEM ($n = 5$). *$P < 0.01$ *versus* all groups; #$P < 0.05$ *versus* OVX and OVX plus low-dose 17β-E_2; †$P < 0.05$ *versus* all groups.

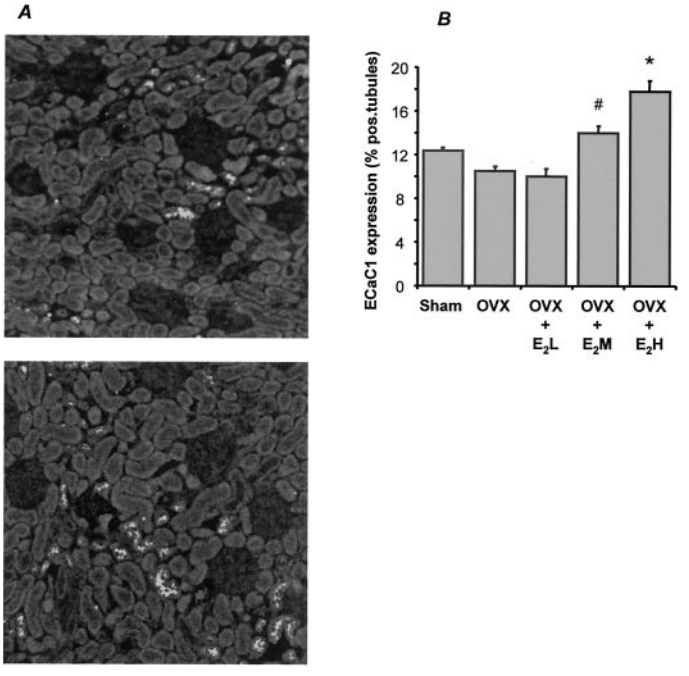

Figure 2. Effects of OVX and 17β-E_2 supplementation on the protein expression levels of ECaC1 in rat kidneys. (A) Immunoperoxidase staining for ECaC1 in kidney cortex sections from OVX rats (top) and OVX/high-dose 17β-E_2-treated rats (bottom). (B) Protein expression levels, calculated by counting the relative amounts of positive tubules in 10 to 15 views in each immunostained section. +E_2L, supplemented with 2 × 32 μg 17β-E_2/d; +E_2M, supplemented with 2 × 125 μg 17β-E_2/d; +E_2H, supplemented with 2 × 500 μg 17β-E_2/d. Data are presented as means ± SEM ($n = 4$). *$P < 0.001$ *versus* all groups; #$P < 0.01$ *versus* OVX and OVX plus low-dose 17β-E_2.

body weights (Table 3), compared with wild-type animals (>20 g) (14,19). After 17β-E_2 treatment, no significant change in body weight was observed. The 1α-OHase-knockout mice exhibited severe hypocalcemia, with plasma Ca^{2+} levels of 1.63 ± 0.06 mM. Surprisingly, after treatment with 17β-E_2, plasma Ca^{2+} levels significantly increased to 2.03 ± 0.12 mM. Measurement of serum 17β-E_2 levels confirmed the increase in 17β-E_2 levels after treatment (Table 3).

Table 3. Effects of 17β-E_2 in 1α-OHase-knockout mice[a]

	Control ($n = 4$)	17β-E_2 ($n = 4$)
Body weight (g)		
beginning	15 ± 2	16 ± 1
final	15 ± 2	14 ± 1
Serum level		
17β-E_2 (pg/ml)	ND	67 ± 12
Ca^{2+} (mM), before	1.54 ± 0.06	1.63 ± 0.06
Ca^{2+} (mM), after	1.69 ± 0.10	2.03 ± 0.12[b]

[a] 1α-OHase, 25-hydroxyvitamin D_3-1α-hydroxylase; control, 1α-OHase-knockout mice; 17β-E_2, 1α-OHase-knockout mice supplemented with 10 μg 17β-E_2/d; ND, not detectable. Data are presented as mean ± SEM.

[b] $P < 0.05$ *versus* all (before and after).

1,25(OH)$_2$D$_3$-Independent Effect of 17β-E_2 on ECaC1 Expression

To investigate the involvement of 1,25(OH)$_2$D$_3$ in the stimulatory effect of 17β-E_2 supplementation on the expression of ECaC1 and other renal Ca^{2+}-transport proteins, control and 17β-E_2-treated 1α-OHase-knockout mice were used. A 2.5-fold increase in ECaC1 transcript levels in the kidney cortex of 17β-E_2-treated mice, compared with control mice, was observed (Figure 3A). However, in contrast to the upregulation of ECaC1, no significant changes in the expression of calbindin-D_{28K}, NCX1, or PMCA1b were observed after treatment (Figure 3, B to E). Immunohistochemical analyses confirmed the increase in ECaC1 expression, after 17β-E_2 treatment, at the protein level, as indicated by the increased staining in the kidney cortex of 17β-E_2-treated mice, compared with control mice (Figure 4A). Semiquantitative assessment of the relative amounts of immunopositive tubules demonstrated a significant increase in the expression of ECaC1 protein after 17β-E_2 treatment (Figure 4B).

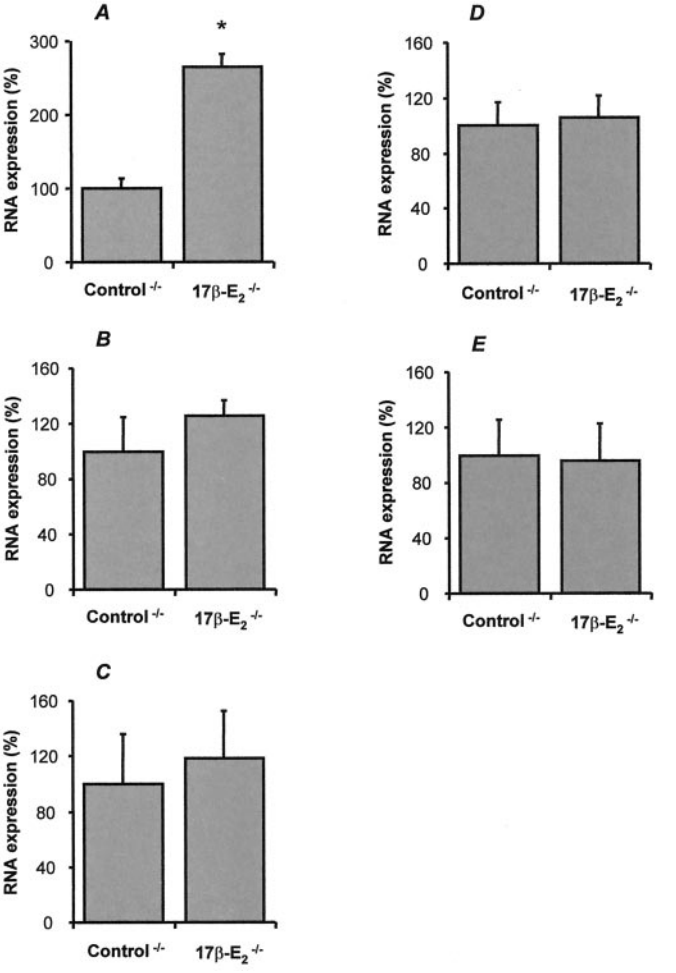

Figure 3. Effects of 17β-E_2 on the mRNA expression levels of ECaC1 and other Ca^{2+}-transport proteins in kidneys from 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase)-knockout mice. The mRNA expression levels of ECaC1 (A), calbindin-D_{28K} (B), calbindin-D_{9K} (C), PMCA1b (D), and NCX1 (E) were measured for the different experimental groups by using real-time quantitative PCR. Control$^{-/-}$, 1α-OHase-knockout mice; 17β-$E_2{}^{-/-}$, 1α-OHase-knockout mice supplemented with 10 μg 17β-E_2/d. Data are presented as means ± SEM ($n = 4$). *$P < 0.001$ *versus* 1α-OHase-knockout mice.

Discussion

The main conclusion from this study is that estrogen upregulates the expression of ECaC1 in the kidney, in a 1,25(OH)$_2$D$_3$-independent manner. By upregulating ECaC1 expression, as the rate-limiting step in transcellular transport, estrogen could be positively involved in Ca^{2+} reabsorption. Estrogen deficiencies after menopause result in bone loss, which is associated with increases in plasma and urinary Ca^{2+} levels (4). It has been generally reported that the increases in plasma and urinary Ca^{2+} levels are secondary to increases in bone resorption. However, some studies have demonstrated that the increases in urinary Ca^{2+} levels after menopause are not attributable to increases in the filtered load, suggesting that estrogen also has an effect on renal Ca^{2+} handling (5,6). This study identified ECaC1 as a molecular target for the action of estrogen in the kidney.

Estrogen replacement therapy for the OVX rats, at pharmacologic doses, resulted in significant upregulation of ECaC1 mRNA and protein expression. ECaC1 protein was observed exclusively at the apical side of distal tubular cells, in agreement with previous studies (17,20). The increased mRNA levels after 17β-E_2 treatment could be attributable to enhanced transcriptional activity or mRNA stabilization. In addition to increases in mRNA abundance, translational regulation of ECaC1 might occur, ultimately resulting in increased channel activity at the apical cell surface. In general, estrogen activates the ER, which subsequently regulates gene transcription by binding to the classic estrogen response elements. ER have been observed in the kidney, especially in the proximal tubules, where 17β-E_2 is retained in the nuclei (10). However, Davidoff *et al.* (9) determined, using autoradiography, that ER are also localized (albeit to a lesser extent) in the distal tubules, the site of ECaC1 expression. Interestingly, Weber *et al.* (21) recently described an estrogen response element in the promoter se-

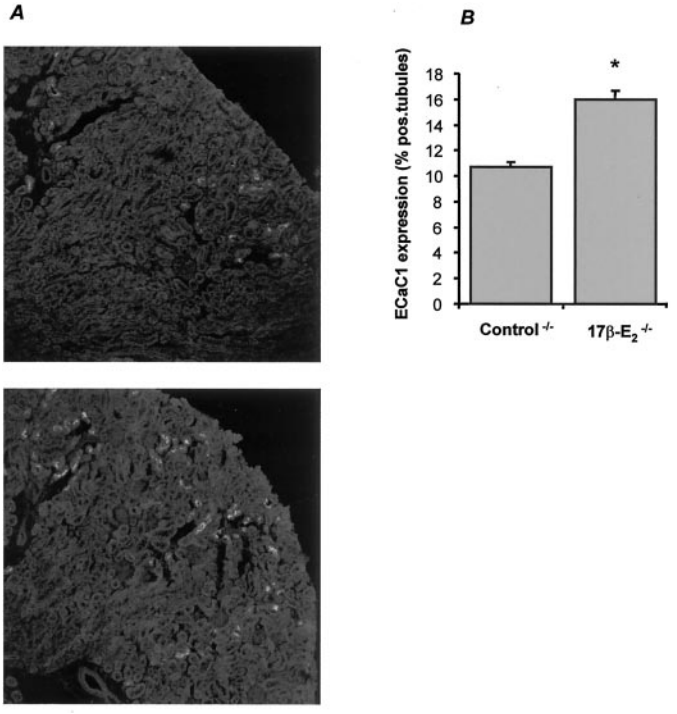

Figure 4. Effects of 17β-E_2 on the protein expression levels of ECaC1 in the kidneys of 1α-OHase-knockout mice. (A) Immunoperoxidase staining for ECaC1 in kidney cortex sections from control (top) and 17β-E_2-supplemented (bottom) 1α-OHase-knockout mice. (B) Protein expression levels, calculated by counting the relative amounts of positive tubules in 10 to 15 views in each immunostained section. control$^{-/-}$, 1α-OHase-knockout mice; 17β-$E_2{}^{-/-}$, 1α-OHase-knockout mice supplemented with 10 μg 17β-E_2/d. Data are presented as means $\pm$ SEM ($n = 4$). *$P < 0.001$ *versus* 1α-OHase-knockout mice.

quence of the mouse ECaC2 gene; the response element was not present in the mouse ECaC1 gene. Alternatively, transcriptional activation by the estrogen-liganded ER can be mediated via activator protein 1 (AP-1) binding sites or GC-rich stimulatory protein (Sp1) binding sites (22,23). Importantly, the human ECaC1 promoter contains several of these AP-1 and Sp1 sites (20,24). Several of these AP-1 and Sp1 binding sites can be observed in the 5'-upstream region of the translation initiation site in the mouse ECaC1 gene and could be involved in the positive effect of 17β-E_2 treatment on ECaC1 mRNA expression. Future studies using ER-knockout mice should indicate whether the observed effects on ECaC1 expression are mediated via ER or an unknown mechanism. Furthermore, detailed promoter analysis is necessary to identify the regulatory sites involved in this estrogen-mediated regulation of ECaC1.

Because the activity of ECaC1 is tightly controlled by cytosolic Ca^{2+} concentrations, a sufficient cytosolic Ca^{2+}-buffering capacity is essential (25,26). This study demonstrates that, in rat kidney, ECaC1 and calbindin-D_{28K} are synchronically upregulated, suggesting that this requirement is fulfilled. There is evidence that an estrogen-responsive promoter exists in the 5'-upstream region of the mouse calbindin-D_{28K} gene (27). Criddle *et al.* (28) demonstrated that estrogen treatment increased renal calbindin-D_{28K} mRNA levels in OVX rats, several hours after administration. In addition, tremendous upregulation of NCX1 and a slight but significant increase in the levels of PMCA1b (both of which are members of the basolateral extrusion system) were observed. In agreement with this finding, it has been suggested that Ca^{2+} extrusion across the basolateral membrane is mediated primarily by NCX1 (13,29). Taken together, these findings suggest a positive effect of 17β-E_2 on renal tubular reabsorption of Ca^{2+}. However, 17β-E_2 treatment resulted in decreased serum Ca^{2+} levels. This effect of estrogen therapy on serum Ca^{2+} levels was previously observed among human subjects (30). This apparent discrepancy can be explained on the basis of the increased Ca^{2+} requirements for estrogen-deficient animals. Correction of estrogen deficiencies results in decreased bone resorption and increased formation, causing slight decreases in plasma Ca^{2+} concentrations (3).

The relationship between estrogen actions in the kidney and vitamin D metabolism is still unclear. $1,25(OH)_2D_3$ is a major regulator of Ca^{2+} homeostasis, and the kidney is the principal site of its synthesis (31). In the literature, conflicting data have been presented regarding direct *versus* indirect effects of 17β-E_2 on Ca^{2+} reabsorption. Some studies demonstrated that estrogen treatment increased $1,25(OH)_2D_3$ levels among postmenopausal women (32). One study demonstrated that 17β-E_2 was retained in the cell nuclei of proximal tubules, where the synthesis of $1,25(OH)_2D_3$ takes place (10). With the use of cultured opossum kidney cells, a suppressive effect of 17β-E_2 on 1α-OHase activity was demonstrated (33). Among postmenopausal women, however, estrogen replacement therapy resulted in unchanged or increased renal 1α-OHase activity (30,34). In previous studies, it was demonstrated that Ca^{2+} reabsorption was stimulated by $1,25(OH)_2D_3$ (35,36). Furthermore, $1,25(OH)_2D_3$ enhances the renal expression of ECaC1 and Ca^{2+}-transport proteins (calbindin-D28k, NCX1, and PMCA1b), as demonstrated in previous studies (20,29,36,37). In contrast, some studies suggested a direct effect of 17β-E_2 on Ca^{2+} reabsorption, independent of vitamin D (3,28).

1α-OHase-knockout mice represent an ideal animal model for studies of the effects of 17β-E_2 on transcellular Ca^{2+} transport independent of $1,25(OH)_2D_3$. As demonstrated in this study and reported by the two laboratories that engineered the 1α-OHase-knockout mice, these animals exhibit severe hypocalcemia (14,19). Treatment with 17β-E_2, at pharmacologic doses, results in significant increases in serum Ca^{2+} levels (to subnormal concentrations) and increases in the expression of ECaC1. These findings suggest a positive effect of 17β-E_2 on plasma Ca^{2+} levels, which might be attributable to increased renal Ca^{2+} reabsorption through ECaC1, independently of $1,25(OH)_2D_3$. Therefore, 17β-E_2 could be directly involved in Ca^{2+} reabsorption via upregulation of ECaC1 gene expression in the absence of circulating $1,25(OH)_2D_3$. In contrast, the expression of the other transport proteins remained unchanged, indicating $1,25(OH)_2D_3$ dependence for the regulation of calbindins, NCX1, and PMCA1b. An explanation for this discrepancy could be that, in addition to 17β-E_2, $1,25(OH)_2D_3$ is

required for optimal stimulation of Ca^{2+} transport, eventually leading to normalized Ca^{2+} levels. Another explanation could be that, in addition to upregulated expression of ECaC1, sufficient calbindins and extrusion proteins are present to facilitate active transport, leading to normalization of serum Ca^{2+} levels. This is in accord with previous studies that suggested that ECaC1 represents the rate-limiting step in active Ca^{2+} transport (12,13). Also, the intestine must be considered to contribute to the increase in serum Ca^{2+} levels. It was previously demonstrated that estrogen has a positive effect on intestinal Ca^{2+} absorption, and it has been suggested that this effect is independent of $1,25(OH)_2D_3$ (16,38,39). ECaC1 and its homolog ECaC2 are present in the duodenum, as are other transport proteins necessary for active Ca^{2+} transport (11,17).

In addition to direct effects on bone, estrogen deficiencies might directly affect renal and intestinal Ca^{2+} handling, resulting in a continuing negative Ca^{2+} balance. Furthermore, interactions with other calciotropic hormones in these tissues must be considered. Because of the high prevalence of osteoporosis, intensive investigations have been performed to clarify the underlying pathophysiologic mechanisms. This study provides evidence that 17β-E_2 is positively involved in renal Ca^{2+} reabsorption via the upregulation of ECaC1, an effect independent of $1,25(OH)_2D_3$. Future detailed studies of the cellular mechanisms of estrogen actions in bone, kidney, and intestine should lead to novel insights regarding the treatment of estrogen-associated disorders.

Acknowledgments

We thank Organon Nederland BV for kindly providing kidney tissue from the OVX rat model study. This work was supported by grants from the Dutch Organization of Scientific Research (Grants Zon-Mw 902.18.298 and Zon-Mw 016.006.001) and by Shriners of North America. Dr. St. Arnaud is a Chercheur-Boursier of the Fonds de la Recherche en Santé du Québec.

References

1. Hurwitz S: Homeostatic control of plasma calcium concentration. *Biochem Mol Biol* 31: 41–100, 1996
2. Hoenderop JG, Nilius B, Bindels RJ: Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol* 64: 529–549, 2002
3. Prince RL: Counterpoint: Estrogen effects on calcitropic hormones and calcium homeostasis. *Endocr Rev* 15: 301–309, 1994
4. Young MM, Nordin BEC: Effects of natural and artificial menopause on plasma and urinary calcium and phosphorus. *Lancet* 2: 118–120, 1967
5. Nordin BEC, Need AG, Morris HA, Horowitz M, Robertson WG: Evidence for a renal calcium leak in postmenopausal women. *J Clin Endocrinol Metab* 72: 401–407, 1991
6. Prince RL, Smith M, Dick IM, Price RI, Webb PG, Henderson NK, Harris MM: Prevention of postmenopausal osteoporosis: A comparative study of exercise, calcium supplementation, and hormone-replacement therapy. *N Engl J Med* 325: 1189–1195, 1991
7. Hagenfeldt Y, Eriksson HA: The estrogen receptor in the rat kidney: Ontogeny, properties and effects of gonadectomy on its concentration. *J Steroid Biochem* 31: 49–56, 1988
8. Lim SK, Won YJ, Lee HC, Huh KB, Park YS: A PCR analysis of ERα and ERβ mRNA abundance in rats and the effect of ovariectomy. *J Bone Miner Res* 14: 1189–1196, 1999
9. Davidoff M, Caffier H, Schiebler TH: Steroid hormone binding receptors in the rat kidney. *Histochemistry* 69: 39–48, 1980
10. Stumpf WE, Sar M, Narbaitz R, Reid FA, DeLuca HF, Tanaka Y: Cellular and subcellular localization of $1,25\text{-}(OH)_2$-vitamin D_3 in rat kidney: Comparison with localization of parathyroid hormone and estradiol. *Proc Natl Acad Sci USA* 77: 1149–1153, 1980
11. Hoenderop JG, van der Kemp AW, Hartog A, van de Graaf SF, van Os CH, Willems PH, Bindels RJ: Molecular identification of the apical Ca^{2+} channel in 1,25-dihydroxyvitamin D_3-responsive epithelia. *J Biol Chem* 274: 8375–8378, 1999
12. Hoenderop JG, Muller D, Suzuki M, van Os CH, Bindels RJ: Epithelial calcium channel: Gate-keeper of active calcium reabsorption. *Curr Opin Nephrol Hypertens* 9: 335–340, 2000
13. Hoenderop JG, Willems PH, Bindels RJ: Toward a comprehensive molecular model of active calcium reabsorption. *Am J Physiol* 278: F352–F360, 2000
14. Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St. Arnaud R: Targeted inactivation of the 25-hydroxyvitamin D_3-$1(\alpha)$-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. *Endocrinology* 142: 3135–3141, 2001
15. Bindels RJM, Hartog A, Abrahamse SL, van Os CH: Effects of pH on apical calcium entry and active calcium transport in rabbit cortical collecting system. *Am J Physiol* 266: F620–F627, 1994
16. Colin EM, van den Bemd GJ, van Aken M, Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, van Leeuwen JP: Evidence for involvement of 17β-estradiol in intestinal calcium absorption independent of 1,25-dihydroxyvitamin D_3 level in the rat. *J Bone Miner Res* 14: 57–64, 1999
17. Hoenderop JG, Hartog A, Stuiver M, Doucet A, Willems PH, Bindels RJ: Localization of the epithelial Ca^{2+} channel in rabbit kidney and intestine. *J Am Soc Nephrol* 11: 1171–1178, 2000
18. Fischer RA: *The Design of Experiments*, edited by Boyd OS, Edinburgh, 1935
19. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D: Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: Evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci USA* 98: 7498–7503, 2001
20. Hoenderop JG, Muller D, van der Kemp AW, Hartog A, Suzuki M, Ishibashi K, Imai M, Sweep F, Willems PH, van Os CH, Bindels RJ: Calcitriol controls the epithelial calcium channel in kidney. *J Am Soc Nephrol* 12: 1342–1349, 2001
21. Weber K, Erben RG, Rump A, Adamski J: Gene structure and regulation of the murine epithelial calcium channels ECaC1 and 2. *Biochem Biophys Res Commun* 289: 1287–1294, 2001
22. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P: Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* 74: 311–317, 2000
23. Safe S: Transcriptional activation of genes by 17β-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* 62: 231–252, 2001
24. Muller D, Hoenderop JG, Merkx GF, van Os CH, Bindels RJ: Gene structure and chromosomal mapping of human epithelial calcium channel. *Biochem Biophys Res Commun* 275: 47–52, 2000

25. Hoenderop JG, van der Kemp AW, Hartog A, van Os CH, Willems PH, Bindels RJ: The epithelial calcium channel, ECaC, is activated by hyperpolarization and regulated by cytosolic calcium. *Biochem Biophys Res Commun* 261: 488–492, 1999
26. Nilius B, Prenen J, Vennekens R, Hoenderop JG, Bindels RJ, Droogmans G: Modulation of the epithelial calcium channel, ECaC, by intracellular Ca^{2+}. *Cell Calcium* 29: 417–428, 2001
27. Gill RK, Christakos S: Regulation by estrogen through the 5'-flanking region of the mouse calbindin-D_{28K} gene. *Mol Endocrinol* 9: 319–326, 1995
28. Criddle RA, Zheng MH, Dick IM, Callus B, Prince RL: Estrogen responsiveness of renal calbindin-D_{28K} gene expression in rat kidney. *J Cell Biochem* 65: 340–348, 1997
29. van Baal J, Yu A, Hartog A, Fransen JAM, Willems PHGM, Lytton J, Bindels RJM: Localization and regulation by vitamin D of calcium transport proteins in rabbit cortical collecting system. *Am J Physiol* 271: F985–F993, 1996
30. Gallagher JC, Riggs BL, DeLuca HF: Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J Clin Endocrinol Metab* 51: 1359–1364, 1980
31. Zehnder D, Hewison M: The renal function of 25-hydroxyvitamin D_3-1α-hydroxylase. *Mol Cell Endocrinol* 151: 213–220, 1999
32. Cheema C, Grant BF, Marcus R: Effects of estrogen on circulating "free" and total 1,25-dihydroxyvitamin D and on the parathyroid-vitamin D axis in postmenopausal women. *J Clin Invest* 83: 537–542, 1989
33. Henry HL: 25(OH)D_3 metabolism in kidney cell cultures: Lack of a direct effect of estradiol. *Am J Physiol* 240: E119–E124, 1981
34. Civitelli R, Agnusdei D, Nardi P, Zacchei F, Avioli LV, Gennari C: Effects of one-year treatment with estrogens on bone mass, intestinal calcium absorption, and 25-hydroxyvitamin D_3-1α-hydroxylase reserve in postmenopausal osteoporosis. *Calcif Tissue Int* 42: 77–86, 1988
35. Costanzo LS, Sheehe PR, Weiner IM: Renal actions of vitamin D in D-deficient rats. *Am J Physiol* 226: 1490–1495, 1974
36. Glendenning P, Ratajczak T, Dick IM, Prince RL: Calcitriol upregulates expression and activity of the 1b isoform of the plasma membrane calcium pump in immortalized distal kidney tubular cells. *Arch Biochem Biophys* 380: 126–132, 2000
37. Gross M, Kumar R: Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am J Physiol* 259: F195–F209, 1990
38. Arjmandi BH, Hollis BW, Kalu DN: *In vivo* effect of 17β-estradiol on intestinal calcium absorption in rats. *Bone Miner* 26: 181–189, 1994
39. Picotto G, Massheimer V, Boland R: Acute stimulation of intestinal cell calcium influx induced by 17β-estradiol via the cAMP messenger system. *Mol Cell Endocrinol* 119: 129–134, 1996