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"Non-hypercalcemic" analogs of 1α,25 dihydroxy vitamin D augment the induction of creatine kinase B by estrogen and selective estrogen receptor modulators (SERMS) in osteoblast-like cells and rat skeletal organs

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Abstract

We have demonstrated previously that daily treatments for 3 days with the so-called "non-hypercalcemic" analogs of 1α,25 dihydroxy vitamin D in ROS 17/2.8 osteoblast-like cells, stimulate the specific activity of creatine kinase BB (CK), and that such treatment with these analogs followed by a single treatment with gonadal steroids, upregulates responsiveness and sensitivity to estradiol 17β (E_2) for the induction of CK. This study was designed to determine if these same "non-hypercalcemic" vitamin D analogs could upregulate in vivo the response to E_2 and whether substitution of selective estrogen receptor modulators (SERMS) for E_2 would result in the same upregulation. We found that one week or 2 weeks pretreatment of prepubertal rats with vitamin D analogs led to increased induction of CK by E_2 and by the SERMS tamoxifen, tamoxifen methiodide and raloxifene, in epiphysis and diaphysis of the femur but not in the uterus. However, in contrast to their antiestrogenic activity in the uterus, there was no inhibition of E_2 action by the SERMS in skeletal tissues. The induction of mRNA for ckb in ROS 17/2.8 cells by E_2 or SERMS was demonstrated only after vitamin D pretreatment; there was no inhibition of E_2 induction by SERMS. Antagonists of vitamin D dependent calcium transport (transcaltachia) did not inhibit stimulation by vitamin D analogs. These results support the involvement of a nuclear mechanism in the upregulation of induction of CK by E_2, which may be due, in part, to the ability of vitamin D to increase estrogen receptor(s).

1. Introduction

The mechanism of physiological bone turnover, and thus the management of postmenopausal osteoporosis, includes the requirement of appropriate concentrations of the steroid hormone estradiol 17β (E_2) and the secosteroid hormone $1,25(OH)_2D_3$ [1,2]. Although we have presented evidence that pretreatment with vitamin D_3 metabolites upregulated the response to E_2 of skeletal derived cells in culture [3–6] as measured by increased [^{3}H]thymidine incorporation into DNA and increased specific activity of the brain-type isozyme of creatine kinase (CK, the predominant CK isozyme in most rat tissues) the significance of these observations is related to the extent to which they could apply to "non-hypercalcemic" vitamin D analogs in vivo and to the growing number of selective estrogen receptor modulators (SERMS) [7–9] now being developed. Among the mutual interactions between estrogens and vitamin D which have been demonstrated [4–6,10,11] an increased response to E_2 after pretreatment with $1,25(OH)_2D_3$ has been correlated with an increase in estrogen receptors (ER) [6]. To understand further the

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mechanism of the upregulation of E_2 action and to asses its possible therapeutic significance, we have addressed the following questions:

1. Does the upregulation response occur in vivo?
2. Is the effect mimicked by SERMS in vitro and in vivo?
3. Is this effect consistent with increased availability of estrogen receptors ?

In this study, we have extended our work using osteoblast-like osteosarcoma cells, ROS 17/2.8, which contain receptors to estrogen [12], like normal human osteoblasts [13] and vitamin D metabolites [14] and respond to both hormones similarly to hormone responsive cells of humans and rats [15–17]. We have also used new "non-hypercalcemic" analogs of vitamin D [18]: hexafluorovitamin D, and the side chain modified derivatives (Fig. 1) EB 1089 [19], CB 1093 and MC 1288, since treatment of ROS 17/2.8 cells by daily additions of these analogs caused a 2–3 fold increase in CK activity and upregulated the response to E_2 [20]. Pretreatment with vitamin D analogs also increased the sensitivity to E_2 [4,5] by lowering the dose for a comparable response to E_2 (by 1–2 orders of magnitude) [20,21]. This increase of CK activity by vitamin D analogs was paralleled by an increase in mRNA for ckb [22]. We also have reported previously that selective estrogen receptor modulators (SERMS); tamoxifen, raloxifene [8] and tamoxifen methiodide [23] stimulate CK activity in ROS 17/2.8 cells when given alone, but antagonize E_2 action when given together with E_2 [23].

In the present study, using stimulation of CK specific activity as a sensitive and rapid post receptor response marker [2–6,23–25], we studied the effect of

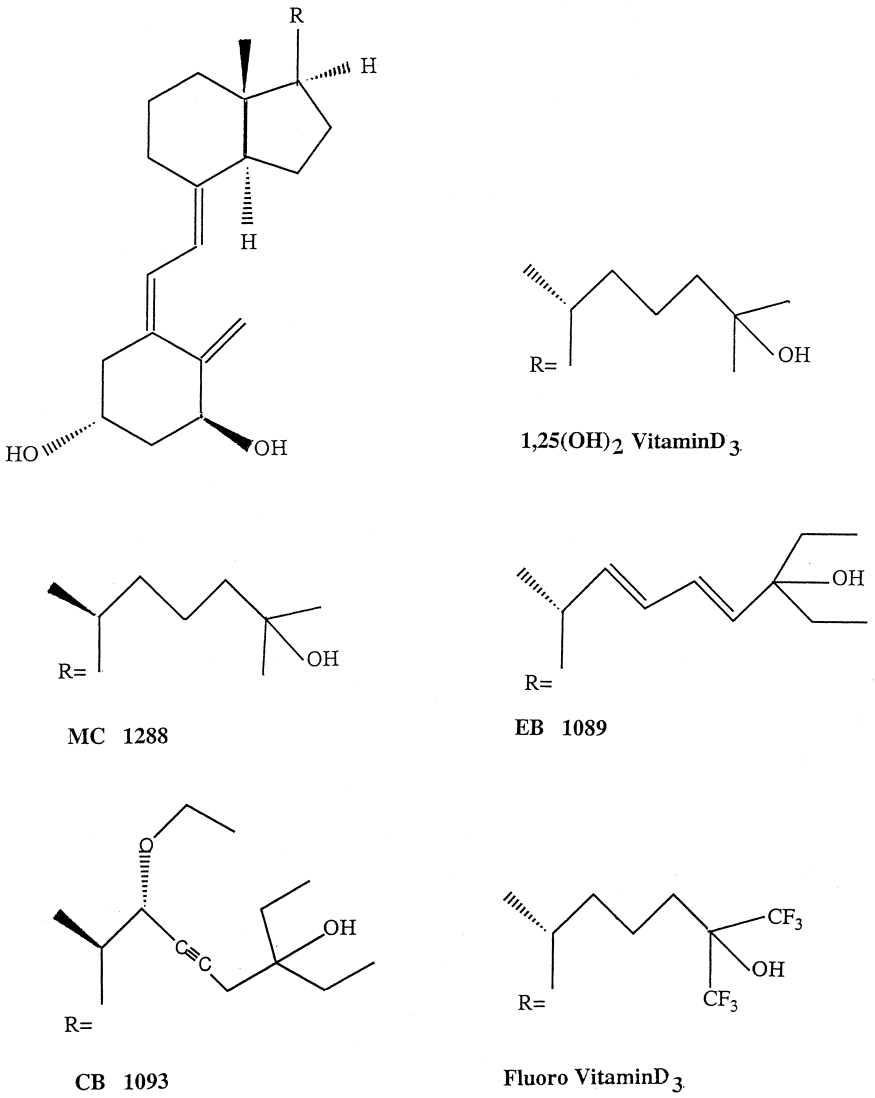

Fig. 1. Structures of $1,25(OH)_2$ vitamin D_3 and the so called "non-hypercalcemic" analogs tested.

vitamin D analog pretreatment on the agonistic and antagonistic effects of SERMS both in vivo and in vitro, and the effects of inhibitors of vitamin D action on the upregulation phenomenon. We found in vivo organ specific upregulation by E_2 that was mimicked by SERMS and evidence consistent with a mechanism involving increased estrogen receptor availability.

2. Materials and methods

2.1. Reagents

Estradiol 17β(E_2) and the UV spectrophotometric creatine kinase assay kit were purchased from Sigma. Tamoxifen (TAM) was the gift of Dr. A. Wakeling (Xeneca, Macclesfield, UK). Tamoxifen methiodide (TMI) was the gift of Dr. A. Beigon (Pharmos, Rehovot, Israel). Raloxifene (RAL) was the gift of Dr. B. Fournier (Ciba-Geigy, Basel, Switzerland). Hexafluorovitamin D was the gift of Sumitomo Pharmaceutical, Osaka, Japan. 1,25$(OH)_2D_3$, CB 1093, EB 1089 and MC 1288 were gifts of Leo Pharmaceutical Products, Ballerup, Denmark. 1β,25$(OH)_2D_3$ (HL) and 2-chloro 1β,25$(OH)_2D_3$ (HS) were gifts of Prof. H. Mayer (GBF, Braunschweig, Germany). All other reagents used were of analytical grade.

2.2. Cell cultures

Fetal calf serum (FCS) was obtained fom Biological Industries, Kibbutz Beit Haemek, Israel. Media were prepared by the Biological Services of the Weizmann Institute of Science. Osteoblast-like ROS 17/2.8 osteosarcoma cells [26] were cultured in 24 well plates in 1 ml of DMEM + F12 (1:1) containing 10% FCS, in a humidified 7.5% CO_2 atmosphere.

2.3. Animals

Female, Wistar-derived, locally bred rats were used at the age of 25 days at a weight of about 60 g. The rats were maintained at 23°C, on a 14 h light, 10 h dark schedule and fed pelleted food and water ad libidum. Experiments were carried out according to NIH guidelines.

2.4. Hormone treatment

1. Cells were treated, starting day 1 after seeding, with three daily additions of vehicle (0.02% ethanol in medium) or 1,25$(OH)_2D_3$, hexaflouorovitamin D, EB 1089, MC 1288 or CB 1093, either at 1 pM or at 1 nM final concentration [18,19] (Fig. 1). On day 4 after seeding, cells were challenged with 30 nM E_2 or 3 μM TAM, RAL, TMI or one of the above SERMS in the presence of E_2. In other experiments, vitamin D analogs were tested in the presence of the vitamin D inhibitors HL or HS at 1 nM. Exposure to E_2 or the SERMS was for 4 h for assaying enzyme activity and 2 h for determining the steady state level of ckb mRNA.
2. Rats were injected with vehicle (0.05% ethanol in saline) or CB 1093, MC 1288 or EB 1089 at 1.875 pg/g initial weight or 1,25$(OH)_2D_3$ or hexaflourovitamin D at 250 pg/g initial weight. The analogs were injected daily for 4 days followed on day 5 by vehicle, or E_2 at 0.5 or 5 μg/rat, SERMS at 500 μg/rat or the SERMS together with 5 μg E_2 per rat for 4 h. In other experiments, vehicle, CB 1093, MC 1288, or EB 1089 at 1.875 pg/g initial weight were injected daily on days 1–5 and 8–11 and E_2 on days 6 and 12; rats were killed 4 h after the injection of E_2 on day 12.

2.5. CK preparation and assay

Cells were scraped from culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction medium [25]. Supernatant extracts were obtained by centrifugation of homogenates at 14,000 *g* for 5 min at 4°C in an Eppendorf micro-centrifuge. Rat organs were collected in cold isotonic extraction buffer [25], homogenized in a Polytron homogenizer (Kinematica A.G.) and enzyme extracts obtained as from the cells (see above). CK activity was measured in a Kontron model 922 Uvicon Spectrophotometer at 340 nm using a Sigma coupled assay kit. Protein was assayed by Coomassie brilliant blue dye binding [27], using BSA as the standard.

2.6. ckb mRNA

ckb mRNA was extracted and analyzed by Northern blot hybridization as previously described [28]. RNA was extracted and 20 μg samples were subjected to hybridization with the plasmid ckb-1, kindly provided by Dr. P. Benfield, and subsequently with an 18S ribosomal RNA probe for normalization of the amounts of total RNA applied [28].

2.7. Statistical analysis

The significance of differences between experimental and control values was evaluated using ANOVA in which n = number of culture dishes or animals.

3. Results

3.1. Upregulation of estradiol induction of creatine kinase in immature female rat organs by "non-hypercalcemic" analogs of vitamin D

In order to evaluate in vivo the biological significance of our findings that pretreatment with "non-hypercalcemic" vitamin D analogs could increase the response of osteoblast-like cells to estrogen [20], we treated prepubertal female rats with four daily doses of vitamin D analogs followed by challenge with 17β estradiol (E_2) on day 5. In contrast to our findings on ROS 17/2.8 osteoblast-like osteosarcoma cells in vitro [20], vitamin D analogs alone did not significantly increase creatine kinase (CK) specific activity in the epiphysis or the diaphysis while significantly stimulating CK activity in the uterus (1.6 fold by CB 1093, 2.2 fold by EB 1089 and 2.3 fold by MC 1288). Under these conditions, in which 0.5 and 5 μg of E_2 alone stimulated CK specific activity by 1.3 and 1.5 fold in epiphysis, 1.6 and 1.9 fold in diaphysis and 1.7 and 2.2 fold in the uterus, pretreatment with the vitamin D analogs MC 1288 and CB 1093 produced, in the epiphysis and in the diaphysis, additional 2.6 to 3.0 fold increases when followed by challenge with 0.5 μg E_2/rat (Fig. 2). EB 1089 produced a modest but significant 1.5 fold augmentation of E_2 stimulation in epiphysis and no increase in diaphysis. In the uterus, none of the three vitamin D analogs caused any increase in estrogen's action (Fig. 2). A higher dose of E_2, did not further increase CK activity; in fact, after MC 1288 pretreatment, challenge with 5 μg E_2 resulted in a smaller augmentation in diaphysis than with 0.5 μg E_2.

Since repeated or continuous use of vitamin D analogs would be necessary for any long term intervention, a 12 day experiment, incorporating a second round of vitamin D analog treatment followed by E_2 challenge was initiated. This second round of

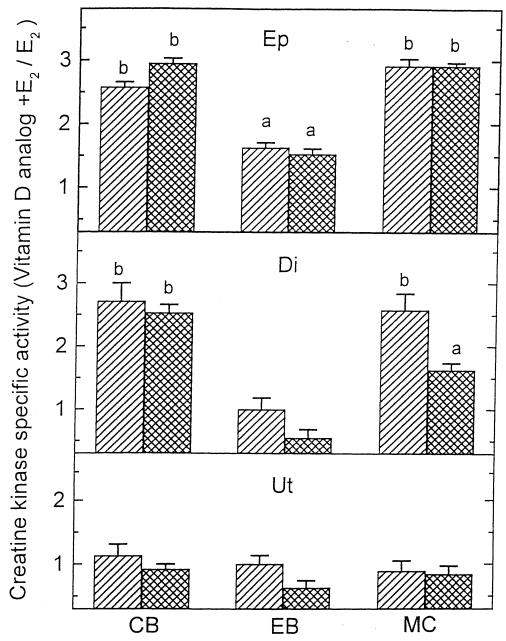

Fig. 2. Organ specific stimulation of CK activity by pretreatment of prepubertal rats with "non-hypercalcemic" analogs of vitamin D, followed by 17β estradiol (E_2). Rats were injected daily for 4 days with vehicle, the side chain analogs (Fig. 1) CB 1093 (CB), EB 1089 (EB) and MC 1288 (MC) at a dose of 1.875 pg/g initial body weight and on day 5 with either 0.5 μg E2/rat (lined bars) or 5 μg E_2/rat (cross hatched bars) and killed 24 h later. Samples of epiphysis (Ep), diaphysis (Di) and uterus (Ut) were collected, homogenized and extracts were prepared and assayed for CK activity as described in Section 2. Results are means ± SEM, for $n = 8–20$, of the ratio of specific activity of CK in rats treated with E_2 after vitamin D compared to rats treated with E_2 after vehicle alone. Means of sequential treatment with a vitamin D analog and E_2 were compared to means of CK specific activity produced by the corresponding E_2 dose after vehicle alone, using ANOVA: a = $P < 0.05$, b = $P < 0.001$.

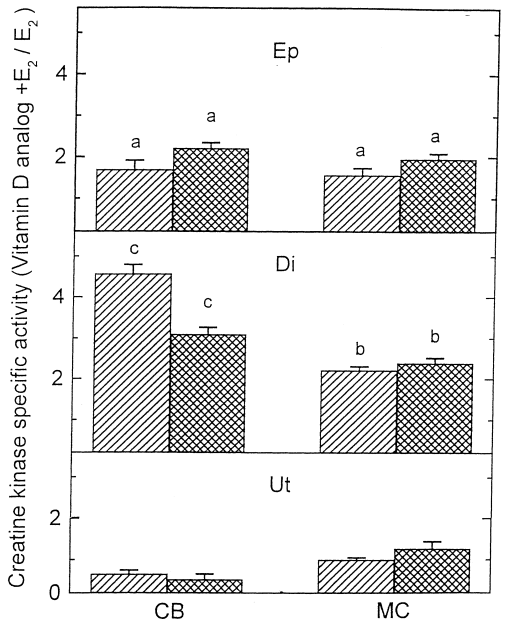

Fig. 3. Organ specific stimulation of CK activity by two rounds of pretreatment of prepubertal rats with "non-hypercalcemic" analogs of vitamin D, (Fig. 1) CB 1093 (CB) or MC 1288 (MC), at a dose of 1.875 pg/g initial weight on days 1–5 and 8–11 and with 0.5 μg E_2/rat (lined bars) or 5 μg E_2/rat (cross hatched bars) on days 6 and 12 and killed 4 h after the injection of E_2 on day 12. Samples of epiphysis (Ep), diaphysis (Di) and uterus (Ut) were collected, homogenized and extracts were prepared and assayed for CK activity as described in Section 2. Results are means ± SEM for $n = 8–16$ of the ratio of specific activity of CK in rats treated with E_2 after vitamin D compared to rats treated with E_2 after vehicle alone. The basal specific activity of CK in Ep was 2.39 ± 0.41, in Di, 2.07 ± 0.18 and in Ut, 3.44 ± 0.1 μmol/min/mg protein. Experimental means were compared to control means of CK specific activity, produced by the corresponding dose of E_2 after vehicle alone, using ANOVA: a = $P < 0.05$, b = $P < 0.001$, c = $P < 0.005$.

analog treatment was poorly tolerated. Rats injected with MC 1288 or CB 1093 lost weight and those treated with EB 1089 died before the experiment was completed. The responses of the control groups injected twice with E_2 were similar to the comparable group (see above) receiving single doses of E_2; i.e. a slightly greater increase in epiphysis (1.6 to 2.1 fold) but a slightly smaller increase in diaphysis (0.97 to 1.4 fold) and in uterus (1.4 to 1.6 fold) for doses of 0.5 and 5 μg/rat, respectively. Both vitamin D analogs, by themselves, caused no significant increase in CK specific activity but did augment the response to E_2 in epiphysis (Fig. 3) although to a lesser degree than after a single treatment. In the diaphysis, CB 1093 was more potent than after single pretreatment. In the uterus, results of two pretreatments (Fig. 3) were similar to that of one pretreatment (Fig. 2).

The toxic effects seen during the double pretreatment have led us to place quotation marks around the term "non-hypercalcemic" as applied to the analogs tested in this study (see Section 4) and to use the term "less calcemic" in the experiments described below.

3.2. Upregulation of CK specific activity by selective estrogen receptor modulators (SERMS) following pretreatment with "less calcemic" vitamin D analogs

A parallel approach to the use of vitamin D analogs to reduce the concentration of estrogens needed as an effective intervention against osteoporosis, is the use of SERMS whose selective action gives them an advantage over estradiol. Therefore, we tested tamoxifen, tamoxifen methiodide and raloxifene, which we showed previously to share the anabolic activity of E_2 to induce CK [23], for their potency after vitamin D analog pretreatment of osteoblast-like cells. Each of the SERMS was capable of significantly stimulating CK activity in ROS 17/2.8 cells to a similar extent as E_2 (Fig. 4, panel +C); this stimulation was prevented in the presence of added E_2. Pretreatment with $1,25(OH)_2D_3$, hexafluorovitamin D, CB 1093, EB 1089

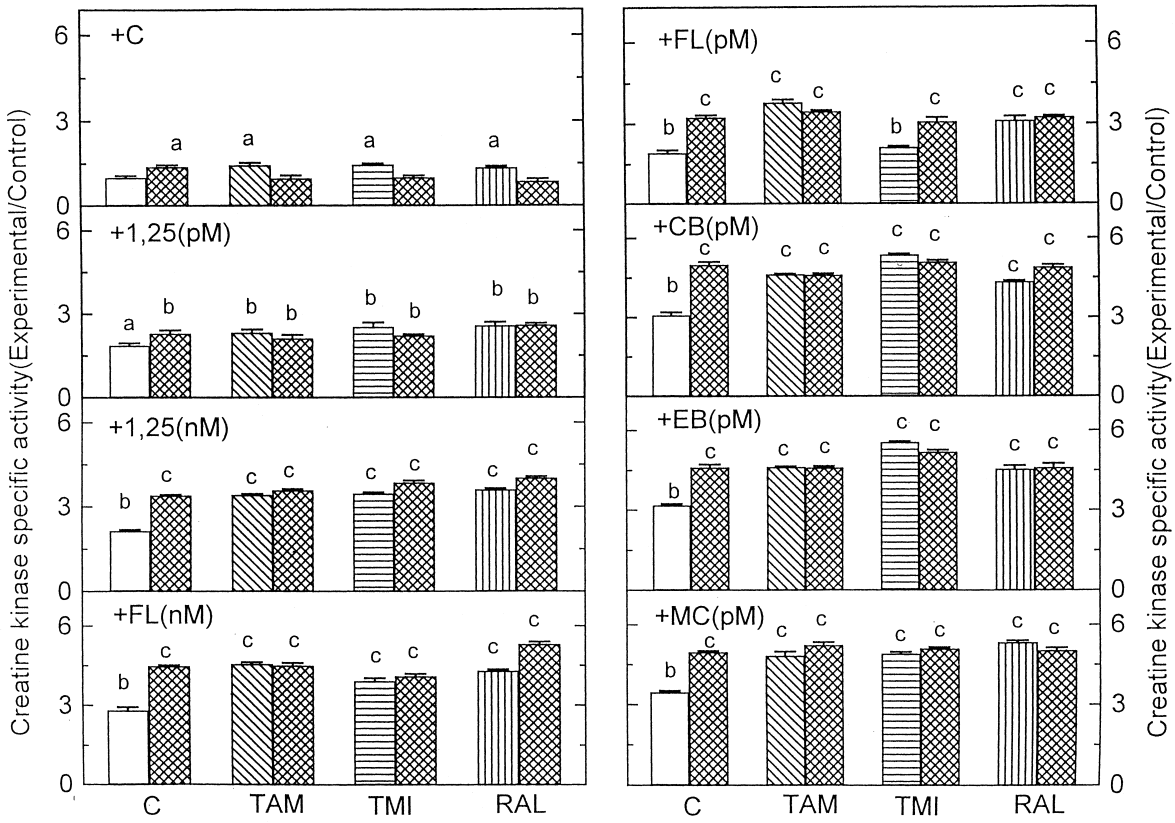

Fig. 4. Stimulation by vitamin D analogs, E_2, SERMS, or their combination, of CK specific activity in ROS 17/2.8 cells. Cultures were treated for 3 days with daily additions of vehicle (C), $1,25(OH)_2D_3$ [1,25], hexafluorovitamin D (FL), CB 1093 (CB), EB 1089 (EB) or MC 1288 (MC). On day 4, the cells were treated for 4 h with control vehicle (C, open bars), 30 nM E_2 or 3 μM selective estrogen receptor modulator (SERM, lined bars): tamoxifen (TAM), tamoxifen methiodide (TMI) or raloxifene (RAL), as well as the combination of E_2 plus a SERM (cross hatched bars). CK extracts were prepared and the specific activity was assayed as described in Section 2. Results are means ± SEM for $n = 8-12$. Untreated control activity was 0.57 ± 0.08 μmol/min/mg protein. Experimental means were compared to control (vehicle alone) mean by ANOVA: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

or MC 1288 highly and significantly increased CK activity and also increased estrogen-like augmentation of CK induction by all three SERMS tested (Fig. 4). But, whereas, after vehicle pretreatment, the SERMS when given together with E_2 abolished E_2 stimulated CK induction, pretreatment with vitamin D analogs prevented inhibition of E_2 action by SERMS (Fig. 4).

For experiments in vivo, prepubertal female rats were injected daily for 4 days with 250 pg hexaflouorovitamin D/g BW, followed by injection on day 5 with 5 μg E_2 or 500 μg SERM, or the combination of both, for 4 h. The organ specific responses to the SERMS (Fig. 5) paralleled the responses to E_2 described above (Fig. 4). In vehicle injected animals (+C), E_2 and all three SERMS increased CK in epiphysis and diaphysis (Fig. 5) while the SERMS antagonized the stimulation of CK activity by E_2, as found above (cf. Fig. 4). In the uterus, only E_2 and TAM were agonistic while TMI and RAL were exclusively antagonistic (Fig. 5).

Pretreatment with hexafluorovitamin D did not increase CK specific activity in any of the organs, but increased the response of epiphysis and diaphysis to E_2 and all the SERMS (Fig. 5). In the uterus, there was no apparent effect of hexafluorovitamin D on the response to E_2 or to TAM (Fig. 5). Hexafluorovitamin D pretreatment prevented all three SERMS, when given together with E_2, from blocking E_2 stimulation of CK activity in epiphysis or in diaphysis; the antagonistic effect of SERMS on E_2 in uterus was unchanged (Fig. 5).

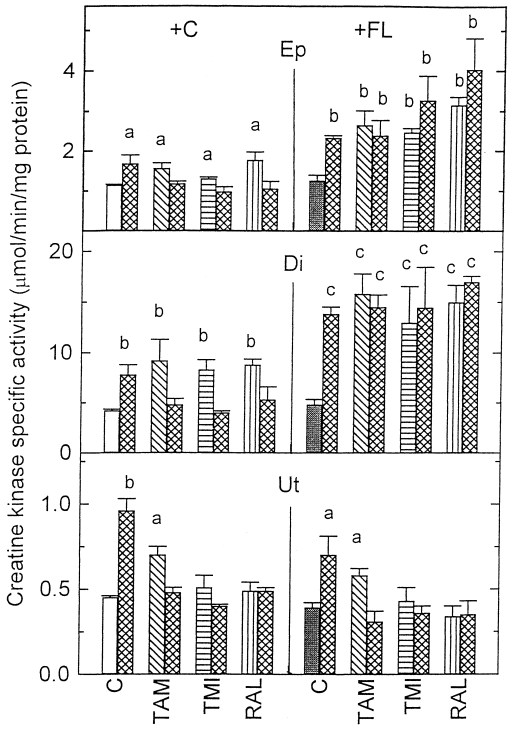

Fig. 5. Organ specific stimulation of CK activity by E_2, by SERMS and by their combination. Prepubertal female rats were given three daily injections of vehicle (C) or hexaflourovitamin D (FL, 250 pg/g BW) followed on day 4 by 5 μg E_2 or 500 μg SERMS (lined bars) or their combination (cross hatched bars) for 4 h. Samples of epiphysis (Ep), diaphysis (Di) and uterus (Ut) were collected and homogenized. Extracts were prepared and assayed for CK as described in Section 2. Results are means ± SEM for $n = 5 - 10$. Experimental means were compared to control means by ANOVA: $a = P < 0.05$; $b = P < 0.01$; $c = P < 0.001$.

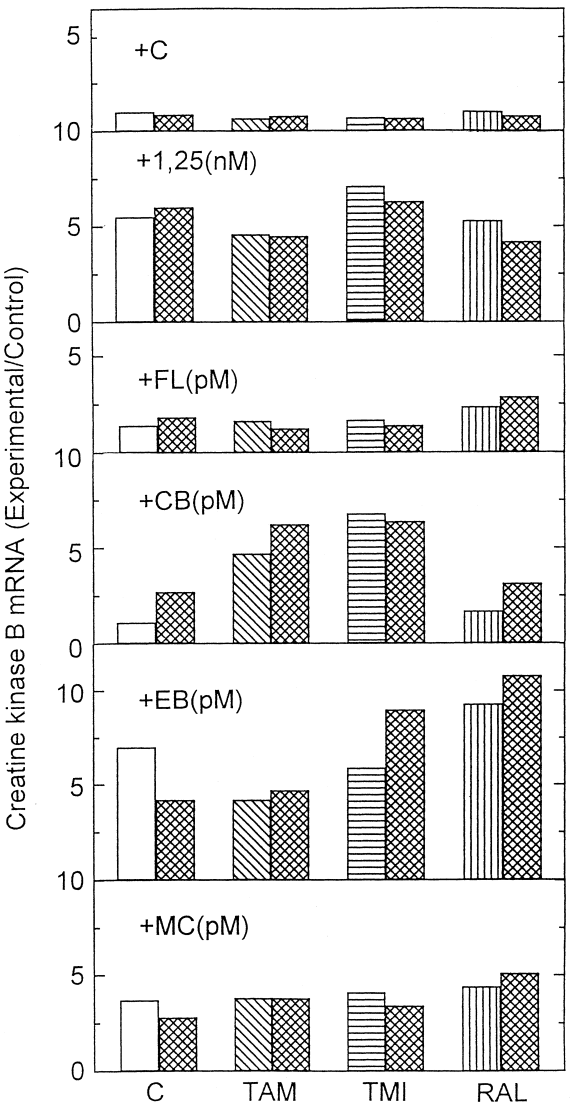

Fig. 6. Vitamin D analogs stimulate the steady state level of ckb mRNA. ROS 17/2.8 cells were treated for 3 days with daily additions of vehicle (C), $1,25(OH)_2D_3$ [1,25], hexafluorovitamin D (FL), CB 1093 (CB), EB 1089 (EB) or MC 1288 (MC). On day 4, the cells were treated for 2 h with control vehicle, 30 nM E_2 or 3 μM SERM (lined bars): tamoxifen (TAM), tamoxifen methiodide (TMI) or raloxifene (RAL), as well as the combination of E_2 and a SERM (cross hatched bars). RNA was extracted and 20 μg samples were subjected to Northern blot hybridization with the plasmid ckb-1, and subsequently with an 18S ribosomal RNA probe, for normalization of the amounts of total RNA applied [28].

3.3. Is upregulation of estrogen receptors (ER) a mechanism of augmentation of E_2 stimulation of CK induction by vitamin D?

The proposed mechanism involving ER [6], implying their involvement in induction of mRNA for CK, and not requiring extranuclear action of vitamin D, was tested in vitro.

When ROS 17/2.8 cells were treated sequentially with vitamin D analogs followed by SERMS, as above, and the steady state level of ckb mRNA was analyzed (Fig. 6), an increase in the level of ckb mRNA detectable by Northern blot hybridization was found only after pretreatment with vitamin D analogs. The stimulation reached up to 10 fold, depending on the vitamin D analog and the SERM, but there was neither consistent inhibition nor stimulation of ckb mRNA when the SERMS were given together with E_2 (Fig. 6).

In contrast to the upregulation of the response of ROS 17/2.8 cells to E_2 after pretreatment with $1,25(OH)_2D_3$, hexafluorovitamin D, MC 1288, EB 1089 or CB 1093 (Fig. 7), two other analogs of $1\alpha,25(OH)_2D_3$, $1\beta,25(OH)_2D_3$ (HL, which is an antagonist of $1,25(OH)_2D_3$-stimulated transcaltachia [29] and of ligands for putative membrane receptors [30]) and 1β,2-chloro-$25(OH)_2D_3$ (HS), were unable to stimulate CK specific activity in the cells. Moreover, these two analogs neither upregulated the response of the cells to E_2, nor did they inhibit upregulation of the E_2 response by any of the analogs (Fig. 7).

4. Discussion

Among the many actions of vitamin D on cells, two separate mechanisms leading to increased creatine kinase activity can be distinguished. One, a direct stimulation of the CK gene evidenced by an increased steady state level of mRNA for CK (Fig. 6) and a sec-

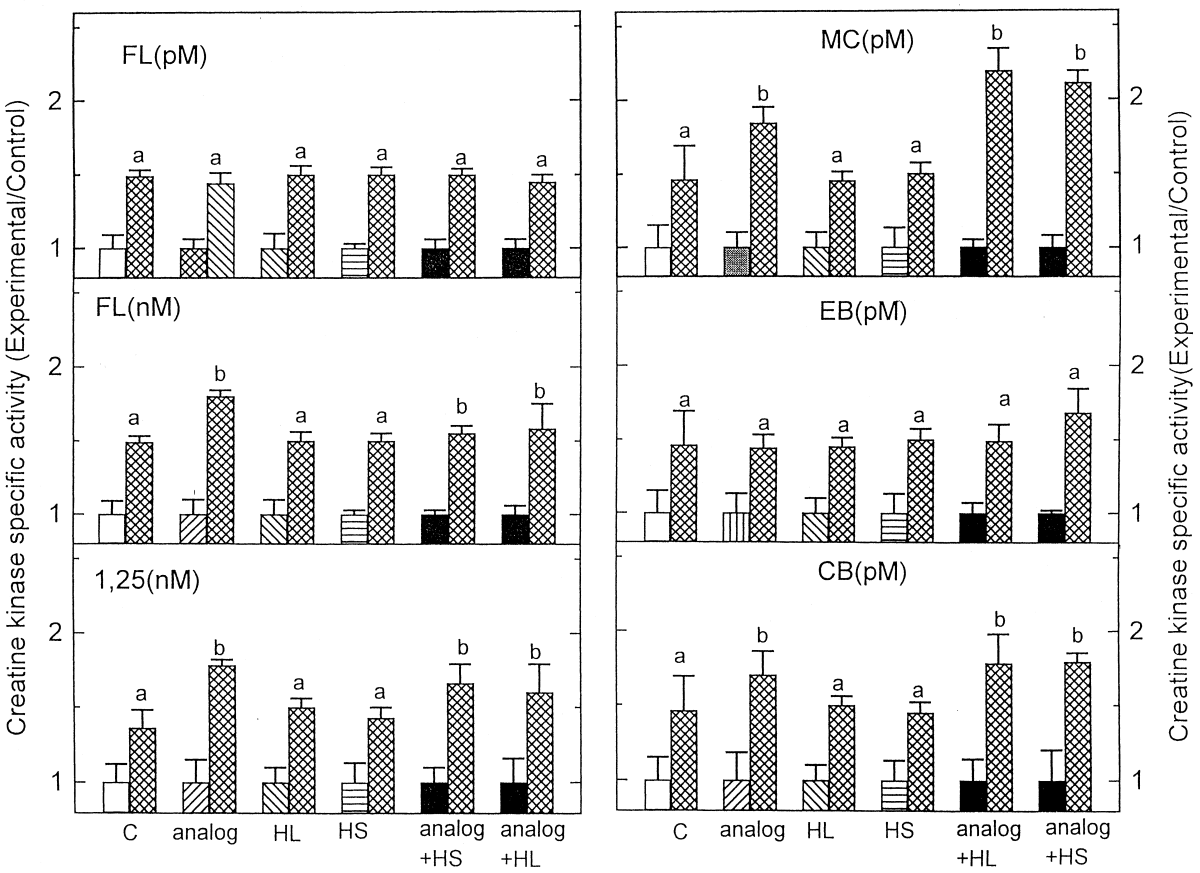

Fig. 7. Inhibitors of $1,25(OH)_2D_3$ induced transcaltachia do not prevent the stimulation of CK specific activity by vitamin D analogs, nor their upregulation of E_2 stimulation. ROS 17/2.8 cells were treated for 3 days with daily additions of $1,25(OH)_2D_3$ [1,25] and the "non-hypercalcemic" analogs (lined bars): hexafluorovitamin D (FL), CB 1093 (CB), EB 1089 (EB), MC 1288 (MC) or the inhibitors of membranal vitamin D action $1\beta,25(OH)_2D_3$ (HL) or 2-chloro-$1\beta,25(OH)_2D_3$ (HS) at 1 nM, or with the combination of the inhibitors with the analogs. On day 4, the cells were treated for 4 h with control vehicle or 30 nM E_2 (cross hatched bars). CK was extracted and assayed as described in Section 2. Results are means ± SEM for $n = 8 - 20$. Untreated control activity was 0.65 ± 0.13 μmol/min/mg protein. Experimental means were compared to control means (analog treated followed by vehicle) by ANOVA: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

ond indirect action via an apparent increase in receptors for estrogen [6,16] which results in upregulation of E_2 induction of CK. This increased responsiveness to estrogens after vitamin D pretreatment, seen previously in ROS 17/2.8 osteosarcoma cells in culture [20–22], has now been demonstrated in prepubertal female rats (Figs. 2 and 3). Although vitamin D analogs did not, by themselves, affect CK in epiphysis, diaphysis or uterus, organ specific upregulation of the responsiveness to E_2 was found in epiphysis and diaphysis but not in uterus (Figs. 2 and 3). This organ specificity of the response to vitamin D analog pretreatment may reflect the much lower concentration of ER in skeletal cells [12,13] which is capable of increase, compared to the ER rich uterus. Since vitamin D metabolites are able to increase the concentration of ER [6,16] in osteoblasts [6] and osteoblast precursor cells [16], supplemental vitamin D could be advantageous in lowering the dose of estrogen required to obtain the optimum anabolic effects of E_2, provided its hyper-calcemic effect [18,19] could be avoided by using ''non-hypercalcemic'' analogs of vitamin D [31–35]. The present attempt to improve the in vivo tolerance to vitamin D by the use of ''non-hypercalcemic'' analogs (Figs. 2 and 3) revealed that they cannot be assumed to be strictly non-hypercalcemic since, in the first attempt to use repeated pretreatment (Fig. 3), rats injected with the analog EB 1089 died during the second round of injections and rats injected with MC 1288 and CB 1093 weighed approximately 30% less than controls at the end of the experiment.

Pretreatment with vitamin D analogs enhanced the response to SERMS in ROS 17/2.8 cells to the same extent as to E_2 (Fig. 4). But surprisingly, after vitamin D analog pretreatment no antagonistic effect was shown by the SERMS [20], on E_2 stimulation of CK in ROS17/2.8 cells [20] or in rat epipyhsis or in diaphysis (Fig. 5). Organ specificity was demonstrated in that, in uterus inhibition by the SERMS of E_2 stimulation of CK was found even after pretreatment with hexaflourovitamin D (Fig. 5) and other vitamin D analogs (data not shown). Organ specificity is also the advantage of the SERMS raloxifene [8] and tamoxifen methiodide [9,23] which do not cause estrogenic hyperstimulation of the uterus leading to endometrial cancer. Upregulation by vitamin D analogs (Figs. 2 and 3) in ER poor skeletal tissues [12,13] but not in ER saturated uterus suggests that the concentration of available ER determines whether upregulation is possible. The mechanism of this upregulation, which was demonstrated in bone marrow-derived stromal cells [16], human bone cell lines Saos-2 [6] and hFOB [36] and in primary human bone cells in culture [36], has been attributed [6] to an increase in the number of estrogen receptors (ER) by $1,25(OH)_2D_3$ in Saos-2 cells [6], in mouse bone marrow-derived cells [16] and in primary cultures of rat skeletal cells [21]. Reciprocally, stimulation of vitamin D receptor levels by E_2 occurs in rat uterus [10] and in Saos-2 cells where estrogen modulates the regulation of osteoblastic function by vitamin D analogs [11,37].

Observations consistent with the proposal that increased ER is the crux of a nuclear mechanism of upregulation are the requirement for three or more days of pretreatment [4], which results in the observed increase in the steady state level of mRNA for ckb (Fig. 6) and the lack of any effect of inhibitors of $1,25(OH)_2D_3$ induced transcaltachia (Fig. 7) on the process.

The organ specificity of the lack of inhibition of E_2 action by SERMS after vitamin D pretreatment (Fig. 5), emphasizes the lack of knowledge concerning estrogen-SERM interactions [23,38]; it raises the question of how these interactions depend on the ratios of E_2 to SERMS, of ERα to ERβ [39,40] and other essential components of the system such as coactivators and corepressors [41,42] which may themselves exhibit organ or cell type specificity. While it has been shown that ERβ mRNA is highly expressed in rat bone [39,40] and in ROS 17/2.8 osteoblast-like cells [40] and even that the ratio of ERα to ERβ changes as the level of ERα mRNA increased during differentiation of osteoblastic cells in culture [40], more data is needed on the ratios of expression of ERα to ERβ in rat organs. This now seems possible using improved immunoassays for ERβ, one of which recently has been used to determine ERβ in human growth plate cartilage [43]. Moreover, the organ specific effects of SERMS must be considered in the light of their selective activities on ERα and ERβ [44,45].

5. Conclusion

In conclusion, these results support the possible use of estrogens at low doses, or organ specific SERMS, in combination with less calcemic analogs of vitamin D [20–22,33–35,37,46] for prevention and/or treatment of metabolic bone diseases such as post-menopausal osteoporosis. To benefit from the lower risks which such a regime might make possible, it will be necessary to obtain new vitamin D analogs with no or much less calciotropic activity, or new analogs with much greater potency that could be used at concentrations even lower than the sub-nanogram doses tested in this study. Experiments are now underway employing additional types of vitamin D analogs [33,35] to attempt to find such compounds and/or to uncover the structure-function relationships [30,46,47] underlying the different specific effects of vitamin D metabolites and analogs which could allow rational design of more useful analogs.

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