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Nongenomic Effects of an Anti-Idiotypic Antibody as an Estrogen Mimetic in Female Human and Rat Osteoblasts

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Abstract We investigated the early effects of the anti-idiotypic antibody (clone $1D_5$), which recognized the estrogen receptor (ER), on cytosolic free calcium concentration ($[Ca^{2+}]i$) and its long term effects on creatine kinase (CK) specific activity in female human and rat osteoblasts. These actions were compared to the known membrane and genomic effects of 17β estradiol (E_2). Like E_2, clone $1D_5$ increased within 5 s $[Ca^{2+}]i$ in both cell types by two mechanisms: 1) Ca^{2+} influx through voltage-gated Ca^{2+} channels as shown by using EGTA, a chelator of extracellular Ca^{2+}, and nifedipine, a Ca^{2+} channel blocker; 2) Ca^{2+} mobilization from the endoplasmic reticulum as shown by using phospholipase C inhibitors, such as neomycin and U-73122, which involved a Pertussis toxin-sensitive G-protein. Clone $1D_5$ and E_2 stimulated CK specific activity in human and rat osteoblasts with ten fold higher concentrations than those needed for the membrane effects (0.1 µg/ml and 10 pM, respectively). Both effects were gender-specific since testosterone and 5α-dihydotesterone were uneffective. Tamoxifen and Raloxifene, two estrogen nuclear antagonists, inhibited CK response to $1D_5$ and E_2 and Ca^{2+} response to $1D_5$, but not Ca^{2+} response to E_2. By contrast, $(Fab')_2$ dimer, a proteolytic fragment of $1D_5$ with antagonist properties, inhibited both membrane and genomic effects of $1D_5$ and E_2. In conclusion, these results imply that clone $1D_5$ has an estrogen like activity both at the membrane and nuclear levels in female human and rat osteoblasts. $1D_5$ must therefore interact with membrane binding sites, penetrate the cells, and reach the nuclear receptors by an as yet uncharacterized mechanism. J. Cell. Biochem. 65:53–66.

Key words: intracellular calcium; Pertussis toxin-sensitive G-protein; phospholipase C; creatinine kinase; gender-specificity; antiestrogens; estrogen mimetic

Gonadal steroids play a crucial role in skeletal development, and postmenopausal estrogen deficiency in humans is associated with increased bone turnover and bone resorption [Heany et al., 1978]. However, the cellular and molecular mechanisms of estrogen action on bone are still poorly understood. Although human [Eriksen et al., 1988] and rat [Komm et al., 1988] osteoblast-like cells bear high-affinity nuclear binding sites for 17β estradiol, the effects of estrogens on osteoblast cell proliferation and functions are still debated.

Estrogens like other steroid hormones exert their major long-term effects on cell growth, differentiation, and function via intracellular receptors to activating some genes [Beato, 1989; Ham and Parker, 1989; Glass, 1994]. These receptors share a common structural and functional organization, with distinct domains that are responsible for ligand-binding, DNA-binding, and transcription activation [Walter et al., 1985; Kumar et al., 1987; Baniahmad and Tsai, 1993; Glass, 1994; Katzenellenbogen, 1996]. However, there is now increasing evidence that steroid hormones interact with components of the cell membrane and may enter their target cells by a membrane-mediated process which does not involve the classic nuclear receptor machinery [Pietras and Szego, 1975; Duval et al., 1983; Batra, 1987; Lieberherr et al., 1989; McEwen, 1991; Blackmore et al., 1991; Gametchu et al., 1991; Lieberherr and Grosse, 1994; Wehling, 1995]. However, the cellular mediators of rapid nongenomic estrogen actions have not been elucidated. The debate now centers around whether membrane estrogen receptors are related to intracellular steroid

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receptors or consist of completely different proteins. In line with the assumption that the membrane and nuclear receptors are structurally related, Pappas and coworkers recently showed that the membrane estrogen receptor shares at least four epitopes with the classic intracellular estrogen receptor [Pappas et al., 1995a,b].

Recently, we reported [Mor et al., 1992] an anti-idiotypic antibody (clone $1D_5$) against anti-estradiol (clone 2F9) that has the capacity of mimicking the various activities of 17β estradiol (E_2) according to the following criteria: (i) the anti-idiotypic antibody recognized the intracellular estrogen receptor (ER) in immunoblotting experiments, and immunoprecipitated the unoccupied ER, but failed to do so using estradiol-occupied ER; (ii) in binding experiments, the anti-idiotypic antibody inhibited the binding of the ligand estradiol to the homologous receptor; (iii) in immunofluorescence studies, the anti-idiotypic antibody recognized estrogen binding sites in cryostat sections prepared from human, rat, and mouse estrogen-responsive tissues; (iv) in dual color flow cytometry, the anti-idiotypic antibody stained the estrogen receptor of thymocytes [Amir-Zaltsman et al., 1993] and monocytes [Mor et al., 1993]; and (v) the anti-idiotypic antibody could mimic the biological activity of estrogens in increasing significantly creatine kinase (CK) specific activity in a gender-specific manner both in vitro in skeletal cells capable of responding to estrogens [Sömjen et al., 1995] and in vivo in rat tissues sensitive to estradiol [Sömjen et al., 1996].

Since estrogens rapidly (less than 5 s) increase cytosolic free calcium concentration in different cell types [Morley et al., 1992; Lieberherr et al., 1993] and inositol 1,4,5-trisphosphate formation via a Pertussis-sensitive G-protein in osteoblasts [Lieberherr et al., 1993], it will be of interest to study in parallel the effects of the estrogen mimetic clone $1D_5$ and its proteolytic fragments as well as estrogens on nongenomic (intracellular calcium) and genomic (CK activity) events in female rat and human osteoblast-like cells.

Creatine kinase (CK) was chosen as a parameter for the long term effects i.e., genomic events since it is an enzyme involved in cellular energy metabolism, buffering and availability, and is known to be a marker for estrogenic activity [Malnick et al., 1983]. Furthermore, CK is closely related to changes in cell division in skeletal-derived cells [Sömjen et al., 1985, 1989, 1995], and is a convenient marker for interaction of E_2 with the ER. Intracellular Ca^{2+} was chosen as a parameter for the rapid effects i.e., nongenomic events since it is a cell messenger playing a central part in cell cycle control, and is an easy marker of transmembrane cell signalling in osteoblasts, the bone-forming cells responsible for the synthesis and mineralization of the bone matrix.

MATERIALS AND METHODS

Animals

2-day-old female Wistar rats were obtained from Charles River (St. Aubin les Elbeufs, France).

Reagents

All reagents used were of analytical grade. 17β estradiol, testosterone, 5α-dihydrotestosterone, Tamoxifen citrate, and Pertussis toxin were from Sigma Chemical Co. (St. Louis, MO). α Minimal Essential Medium (αMEM) without phenol red, fetal calf serum, and trypsin/EDTA were from Gibco BRL (Eragny, France). Fura2/AM was from Amersham (Les Ulis, France). 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrole-2,5- dione (U-73122) and 1-(6-((17β-3-metoxyestra-1, 3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrolidine- 2,5-dione (U-73343) were from Biomol Research Laboratory (Plymouth, MA) and Tebu (Le Perray en Yvelines, France); nifedipine and neomycin were from Research Biochemicals International (Natick, MA) and Bioblock Scientific (Illkirch, France). Biochemicals, mouse serum, and mouse antibody Isotyping Kit were obtained from Sigma Chemical Co. (St. Louis, MO). Sepharose Protein A was from Pharmacia (Uppsal, Sweden). Immobilized-pepsin was purchased from Pierce (Rockford, IL). Raloxifene was a gift from Ciba-Geigy (Bazel, Switzerland).

Antibodies

Clone $1D_5$, a mouse monoclonal antibody, directed against monoclonal anti-estradiol antibody [Barnard and Kohen, 1990] was propagated as ascites in pristane primed mice, and a purified IgG fraction was isolated by chromatography on Sepharose Protein A, as previously described [Barnard and Kohen, 1990]. The $F(ab')_2$ fragment of clone $1D_5$ was prepared by immobilized-pepsin digestion of a purified frac-

tion of clone $1D_5$, followed by affinity chromatography on Sepharose Protein A, as previously described [Sömjen et al., 1995]. Mouse IgG_1 was prepared by binding of normal mouse serum on Sepharose Protein A followed by elution with 0.1 M phosphate at pH 6.0. The heavy chain class of eluted fractions were determined using the Ochternoly double immunodiffusion assay and the Mouse Antibody Isotyping Kit.

Isolation and Cell Culture

Rat osteoblasts were isolated from parietal bones of 2-day-old females by sequential digestion [Wong and Cohn, 1974] and grown either on glass coverslips for calcium measurement or in Petri dishes for CK activity assay in phenol red-free α Minimal Essential Medium (αMEM) containing 10% heat-inactivated fetal calf serum (H-FCS). Human osteoblasts were isolated from trabecular bone obtained from six 15–17-year-old females after bone surgery. Trabecular bone was cut into small pieces and put in calcium-free αMEM with 10% H-FCS until the osteoblasts were released from the bone pieces and reached confluence. Cells were then trypsinized and cultured in αMEM containing 1.8 mM calcium plus 10% H-FCS either on glass coverslips or in Petri dishes. At this stage of culture, the bone cells present the following characteristics: (i) 89 ± 2% (means ± S.E.M., n = 6) of human bone cells stain for alkaline phosphatase; (ii) the osteocalcin concentration measured in serum-free medium is 10-fold increased by 10 nM 1,25-dihydroxyvitamin D_3 (2.1 ± 0.5 ng/ml/10^6 cells (means ± S.E.M., n = 6) vs. 21.3 ± 0.9 ng/ml/10^6 cells (means ± S.E.M., n = 6)) after 24 h incubation; (iii) the Northern blot analysis of total RNA obtained from the cells isolated from the six bone samples shows a good signal for type I collagen, but no signal for type III collagen (data not shown). Collagen type III is not coexpressed with collagen type I in normal osteoblast cell [Scott et al., 1980]. Moreover, the basal synthesis of type I collagen [Peterkofky and Dielgelmann, 1971] in these cells represents 12 ± 3%, (means ± S.E.M., n = 6); (iv) 1 nM bovine parathyroid hormone (bPTH) increases the cellular cAMP content after 5 min (3.45 ± 0.45 pmol/mg protein vs. 35.9 ± 1.2 pmol/mg protein; means ± S.E.M., n = 6); (v) 1 nM bPTH also increases the intracellular calcium concentration in less than 10 s (183 ± 7 nM vs. 309 ± 10 nM; means ± S.E.M., n = 6).

Calcium Measurement and Experimental Protocol

For this study, we used osteoblasts grown on glass coverslips. This technique implies that the whole glass coverslip must totally be covered by the cells to eliminate some artefacts due to the autofluorescence of the glass. So, confluent osteoblasts were used instead of proliferating cells. The advantage of this method is that cell damage is avoided, and cell-cell contact is maintained. Moreover, in the literature, no example showed any difference in the behaviour of growing and confluent osteoblasts concerning the rapid and direct effects of different hormones on the modulation of intracellular calcium responses.

Cells were washed three times with Hanks' Hepes, pH 7.4 (137 mM NaCl, 0.441 mM KH_2PO_4, 0.885 mM $MgSO_47H2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$, 20 mM Hepes), and loaded with 1 µM Fura-2/AM for 40 min in the same buffer at room temperature. The glass coverslip carrying the cells was inserted into a cuvette containing 2.5 ml Hanks' Hepes, pH 7.4. The cuvette was placed in a thermostatted (37°) Hitachi F-2000 spectrofluorometer. Drugs and reagents were added directly to the cuvette under continuous stirring.

The Fura-2 fluorescence response to intracellular calcium concentration ($[Ca^{2+}]i$) was calibrated from the ratio of 340–380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. [1985]. The dissociation constant for Fura-2.Ca^{2+} was taken as 224 nM [Grynkiewicz et al., 1985]. The values for R_{max} and R_{min} were calculated from measurements using 25 µM digitonin and 4 mM EGTA and enough Tris base to raise the pH to 8.3 or higher. Each measurement on Fura-2 loaded cells was followed by a parallel experiment under the same conditions with non-Fura-2 loaded cells.

The following studies were carried out: first, we studied the direct effects of $1D_5$ (0.05 µg/ml–0.3 µg/ml) on $[Ca^{2+}]i$ of confluent rat and human osteoblasts. Second, we investigated whether the action of $1D_5$ on $[Ca^{2+}]i$ was due to an influx of Ca^{2+} from the extracellular milieu and/or Ca^{2+} mobilization from intracellular stores. Two types of blocking experiments were performed. In the first, a small excess of EGTA (2 mM) was added to the cuvette medium [Al-

bert and Tashjian, 1984]. Replenishement of Ca^{2+} to 1.25 mM Ca^{2+} excess following EGTA treatment restored the basal level. In the second, the selective blocker of Ca^{2+} entry, nifedipine, was added to give a final concentration of 1 μM. It induced a decrease in $[Ca^{2+}]i$ by blocking Ca^{2+} influx via active voltage-dependent channels [Albert and Tashjian, 1984]. To examine what part of the $[Ca^{2+}]i$ transient was due to Ca^{2+} release from the intracellular stores, we used two drugs: neomycin, which inhibits phospholipase C via binding to phosphoinositides [Prentki et al., 1986] and, U-73122, a direct inhibitor of the phospholipase C linked to the hydrolysis of phosphatidylinositol 4,5 bisphosphate [Bleasdale et al., 1989].

Creatine Kinase Extraction and Assay

For this study, we used subconfluent cells which can be still stimulated for growth, and therefore the induction of CK and DNA. But still they are differentiated osteoblasts which contain all the necessary receptors.

Cells were scraped from culture dishes, and homogenized by freezing and thawing three times in cold extraction buffer containing 50 mM Tris HCl, pH 6.8, 5 mM magnesium acetate, 2.5 mM dithiothreitol, 0.4 mM EDTA, and 250 mM sucrose. Homogenates were centrifuged at 14,000*g* for 5 min at 5°C. The supernatant extracts were then lyophilized and stored at −20°C. For assay, lyophilizates were dissolved in double distilled water.

CK activity was measured at 340 nm in a Uvicon, Kontron automatic computerized recording spectrophotometer, using a coupled assay (kit obtained from Sigma), in 0.7 ml incubation mixture containing 50 mM imidazole acetate buffer, pH 6.7, 25 mM creatine phosphate, 20 mM N-acetylcysteine, 20 mM D-glucose, 10 nM magnesium acetate, 5 mM EDTA, 2 mM adenine diphosphate, 2 mM nicotinamide adenine dinucleotide (NAD), 2 mM dithiothreitol, 50 μM diadenosine pentaphosphate (adenylate kinase inhibitor), 5 μg bovine serum albumine (BSA), 1.2 U glucose-6-phosphate dehydrogenase (from Leuconostoc, because of its ability to utilize NAD more efficiently than nicotine adenine dinucleotide phosphate), and 0.8 U hexokinase. A unit of enzyme activity was defined as the amount yielding 1 μmole adenosine trisphosphate/min at 30°C, and the specific activity as units/mg protein. Protein was determined by the Bradford method [Bradford, 1976].

Statistical Analysis

The significance of differences between experimental and control means was evaluated by unpaired two-tailed Student's *t*-test. The results are the means ± S.E.M., and a value of n represents six different primary cultures for rat osteoblasts and in different secondary cultures for four to six human donors.

RESULTS

Effects of $1D_5$ on Intracellular Calcium Concentration

The basal level of intracellular calcium concentration was 141 ± 6 nM (means ± S.E.M., n = 6) in confluent female rat osteoblasts, and 183 ± 7 nM (means ± S.E.M., n = 6) in confluent female human osteoblasts.

Figure 1 shows the transient increase in $[Ca^{2+}]i$ induced by 0.2 μg/ml $1D_5$ and 10 pM 17β estradiol in female rat and human cells. $[Ca^{2+}]i$ dropped rapidly after 30 s, but remained higher than the basal level (25 ± 2%, means ± S.E.M., n = 6, $P < 0.001$). The concentration-dependent effects of $1D_5$ and E_2 were bell-shaped, with a maximal activity at 0.2 μg/ml for $1D_5$ and 100 pM for E_2 in both cell types (Fig. 2). Mouse IgG_1, used as control for $1D_5$, and the vehicle (ethanol) of E_2 had no effect on $[Ca^{2+}]i$ (Fig. 2).

10 pM to 10 nM Testosterone had no effect on $[Ca^{2+}]i$ (data not shown).

Mechanisms Involved in $1D_5$- and Estradiol-Induced Changes in Intracellular Calcium Concentration in Female Rat and Human Osteoblasts

To characterize a possible Ca^{2+} influx, 0.2 μg/ml $1D_5$ or 100 pM E_2 (maximal active concentrations) was added 30 s after 2 mM EGTA, a chelator of extracellular calcium or 60 s after 1 μM nifedipine, a Ca^{2+} channel blocker. EGTA and nifedipine not only diminished (50 ± 10%, means ± S.E.M., n = 6, $P < 0.001$) the transient peak induced either by $1D_5$ or E_2, but totally abolished the sustained plateau phase in both cell types (Fig. 3A and Table I).

To characterize a possible Ca^{2+} mobilization from intracellular stores, cells were pretreated for 3 min with 1 mM neomycin, an indirect inhibitor of phospholipase C, with 2 μM

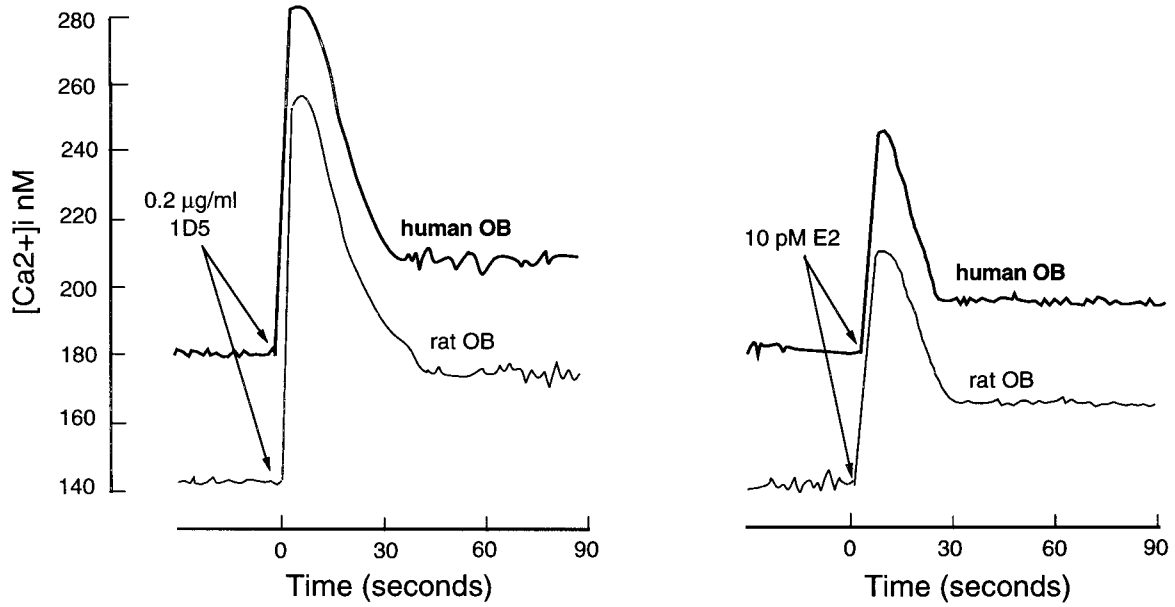

Fig. 1. Effects of 0.2 µg/ml $1D_5$ and 100 pM 17β estradiol on intracellular calcium concentration in confluent female human and rat osteoblasts (OB). These results are representative of at least six different cultures.

U-73122, a direct inhibitor of phospholipase C or with 2 µM U-73343, a closed analog of U-73122 but inactive [Smith et al., 1990], before adding 0.2 µg/ml $1D_5$ or 100 pM estradiol. Neomycin and U-73122 totally abolished the transient spike induced by $1D_5$ or E_2, but did not modify the plateau phase in both cells types (Fig. 3B and Table I). U-73343 (0.3–5 µM) had no effect on intracellular calcium response to $1D_5$ or E_2 (data not shown).

To demonstrate a possible involvement of a G-protein, osteoblasts were pretreated for 16 h

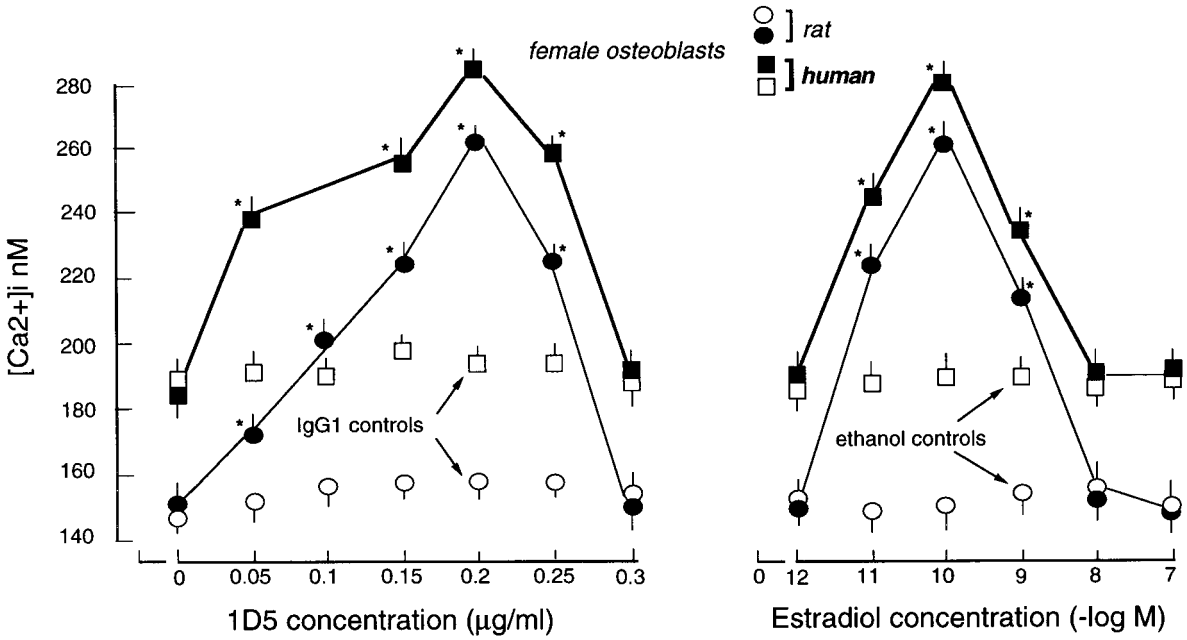

Fig. 2. Dose-dependent effects of $1D_5$ and 17β estradiol on intracellular calcium concentration in confluent female human and rat osteoblasts. Intracellular Ca^{2+} concentrations were determined at 10 s. Values are the means ± S.E.M., n = 6 for each substance and are significantly different from the basal level. *$P < 0.001$.

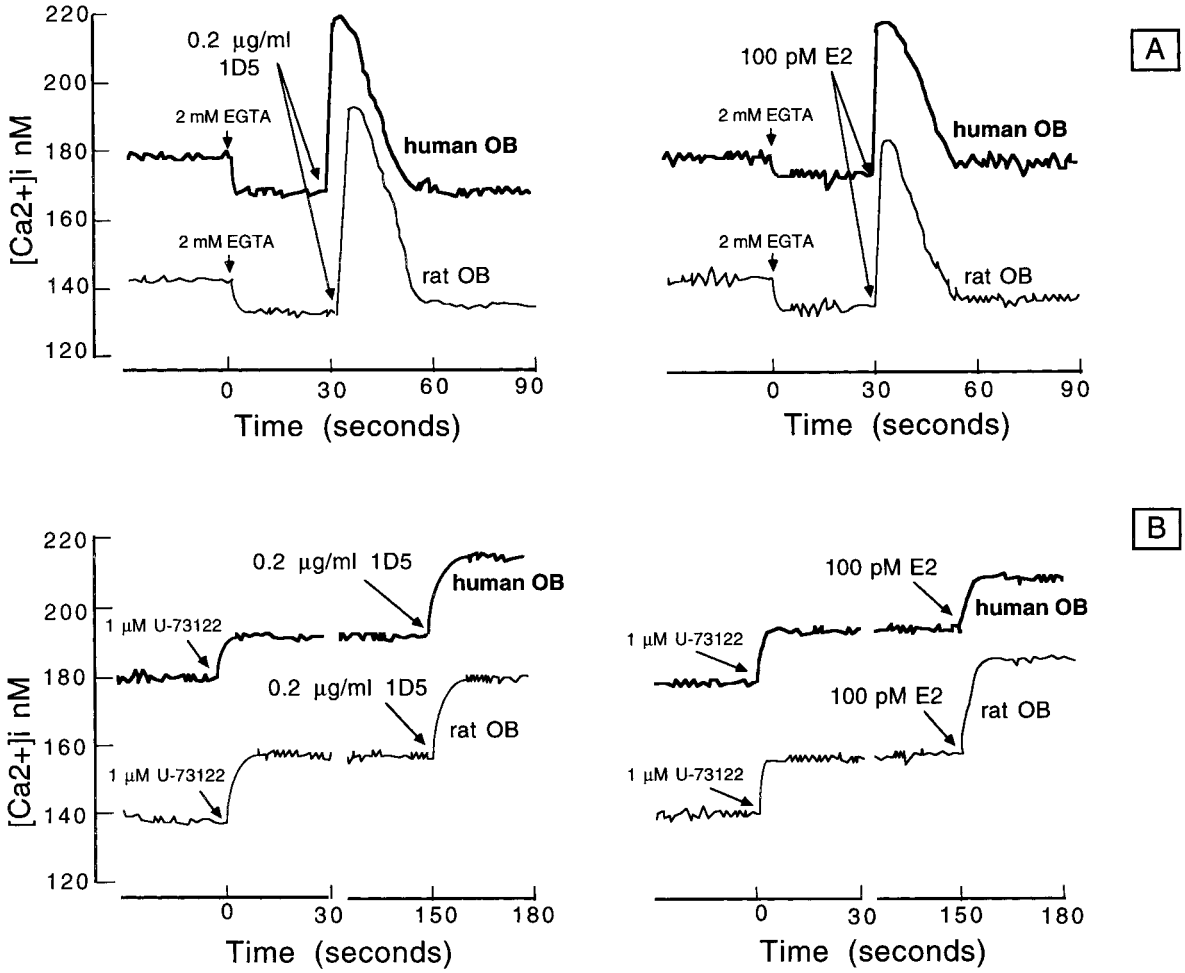

Fig. 3. Effects of EGTA and U-73122 on intracellular calcium response to $1D_5$ or 17β estradiol in confluent female human and rat osteoblasts. **A:** Osteoblasts (OB) were incubated for 30 s with 2 mM EGTA before adding 0.2 µg/ml $1D_5$ or 100 pM 17β estradiol (E_2). **B:** OB were incubated for 3 min with 1 µM U-73122, a direct inhibitor of phospholipase C, before adding 0.2 µg/ml $1D_5$ or 100 pM E_2. These results are representative of at least six different cultures for each experimental case.

with 100 ng/ml of Pertussis toxin (PTX) or 1 µg/ml Cholera toxin (CTX). Fura-2/AM loading and $[Ca^{2+}]i$ measurements were carried out with both toxins. Preincubation with PTX or CTX did not alter the basal level of $[Ca^{2+}]i$. PTX totally abolished the spike phase induced by both 0.2 µg/ml 1D5 and 100 pM E_2 while the plateau phase was unchanged in both cell types (Fig. 4). CTX had no effect (data not shown).

Effects of $1D_5$ and Estradiol on Creatine Kinase Activity in Female Rat and Human Osteoblasts

Figure 5 shows the effects on CK specific activity when cells were incubated for 4 h with different concentrations of $1D_5$ (0.2 to 3.75 µg/ml) or 17β estradiol (0.1–100 nM). The basal level of CK activity was 40 ± 5 nmol/min/mg protein (means ± S.E.M., n = 6) in female rat osteoblasts, and 390 ± 30 nmol/min/mg protein (means ± S.E.M., n = 6) in female human osteoblasts. 1.25 to 3.25 µg/ml $1D_5$ or 1 to 100 nM E_2 increased CK activity in both cell types. The concentration-dependent effects of $1D_5$ and E_2 were bell-shaped in female rat osteoblasts, with a maximal activity at 2.5 µg/ml for $1D_5$ or 10 nM for E_2 (Fig. 5). 2.5 µg/ml $1D_5$ or 10 nM E_2 induced a three-fold increase in CK activity. In contrast, in female human osteoblasts the effects of $1D_5$ or E_2 were not saturable since increasing concentrations of $1D_5$ or E_2 still increased CK activity, with a three-fold increase in CK activity for 3.75 µg/ml $1D_5$ and 100 nM E_2. Neither ethanol nor mouse IgG_1 used as controls had any effect on CK activity. In addi-

TABLE I. Blockade of $1D_5$- and 17β Estradiol-Induced Changes in Intracellular Calcium Concentration in Confluent Female Rat and Human Osteoblasts*

	Female rat osteoblasts			Female human osteoblasts		
	$[Ca^{2+}]i$ nM			$[Ca^{2+}]i$ nM		
		Stimulated level			Stimulated level	
	Basal level	+1 D_5	+E_2	Basal level	+1 D_5	+E_2
	141 ± 6	257 ± 6[a]	262 ± 6[a]	183 ± 6	285 ± 5[a]	286 ± 5[a]
EGTA (2 mM)	143 ± 3	188 ± 5[a,b]	176 ± 7[a,b]	174 ± 6	222 ± 7[a,b]	277 ± 8[a,b]
nifedipine (1 µM)	144 ± 4	181 ± 6[a,b]	181 ± 8[a,b]	181 ± 6	229 ± 7[a,b]	234 ± 8[a,b]
neomycin (1 mM)	132 ± 6	164 ± 4[a,b]	174 ± 6[a,b]	173 ± 6	231 ± 9[a,b]	229 ± 4[a,b]
U-73122 (1 µM)	159 ± 5	187 ± 6[a,b]	181 ± 7[a,b]	192 ± 5	231 ± 4[a,b]	232 ± 5[a,b]

*Values are the means ± S.E.M., n = 6. Osteoblasts were incubated for 30 s with 2 mM EGTA, for 60 s with 1 µM nifedipine, for 3 min with 1 mM neomycin or 1 µM U-73122 before adding 0.2 µg/ml $1D_5$ or 100 pM 17β estradiol (E_2).
[a]= $P < 0.001$ for the difference between treatment with $1D_5$ or E_2 and the basal level.
[b]= $P < 0.001$ for the difference between treatments with blockers and $1D_5$ or E_2 alone. $[Ca^{2+}]i$ were measured at t = 10 s after the addition of $1D_5$ or estradiol.

tion, cells respond only to estrogen since 1 nM 5α-dihydrotestosterone (DHT) had no effect (Fig. 5).

Effects of Anti-Estrogens and $(Fab')_2$ on Intracellular Calcium and Creatine Kinase Response to $1D_5$ and E_2 in Female Rat and Human Osteoblasts

Cells were pretreated for 5 min or 4 h with 1 µM either Tamoxifen or Raloxifene, two anti-estrogens, before adding 0.2 µg/ml $1D_5$ or 100 pM E_2. 1 µM Tamoxifen or Raloxifene alone transiently increased $[Ca^{2+}]i$ within 5 s in both cell types (Fig. 6). After 1 min, the $[Ca^{2+}]i$ returned to the basal level. Four hour incubation with 1 µM Tamoxifen or Raloxifene did not modify the basal level of $[Ca^{2+}]i$ (data not shown). The two antiestrogens were unable to block the estradiol-induced increase in $[Ca^{2+}]i$ in both cell types whatever the incubation time. In contrast, the $1D_5$-induced increase in $[Ca^{2+}]i$ was partially blocked (55 ± 6%, n = 6, $P < 0.001$) by Tamoxifen and Raloxifene in both cell types. This inhibition was more pronounced in female human cells (70 ± 7%, n = 6, $P < 0.001$). Preincubation of the cells for 2 min or 1 h with 2 µg/ml $F(ab')_2$, an antagonist of the anti-idiotypic antibody $1D_5$ obtained by removing the Fc fragment of this antibody [Sömjen et al., 1995], partially blocked the $[Ca^{2+}]i$ response to $1D_5$ and estradiol, with a more accentuated inhibition (15 ± 3%, n = 6, $P < 0.001$) for $1D_5$ in both cell types. This inhibition was the highest in human osteoblasts (Fig. 6). Mouse IgG_2, used as control for $F(ab)_2$, had no effect.

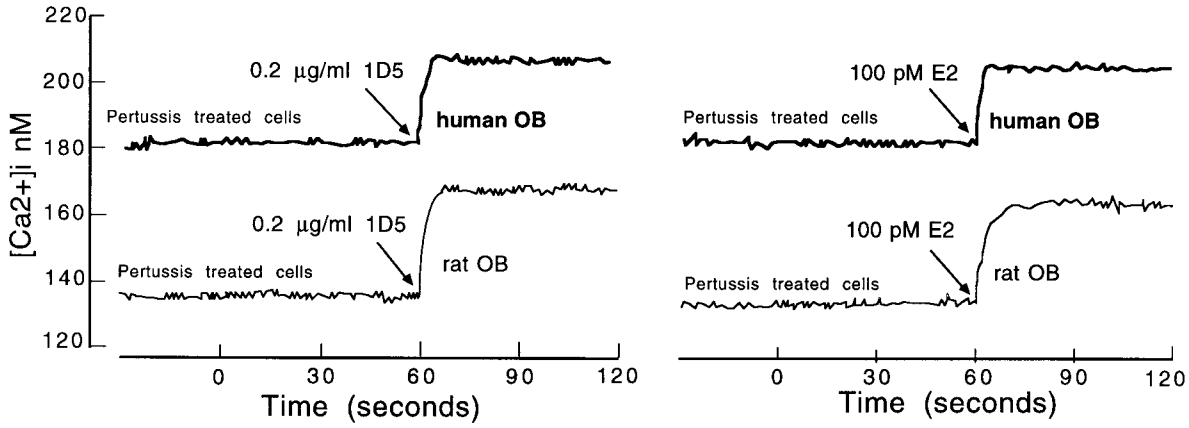

Fig. 4. Effects of Pertussis toxin on intracellular calcium response to $1D_5$ or 17β estradiol in confluent female human and rat osteoblasts. Osteoblasts (OB) were incubated for 16 h with 100 ng/ml Pertussis toxin (PTX). Fura2/AM loading and calcium measurement were done in the presence of PTX. These results are representative of at least six different cultures.

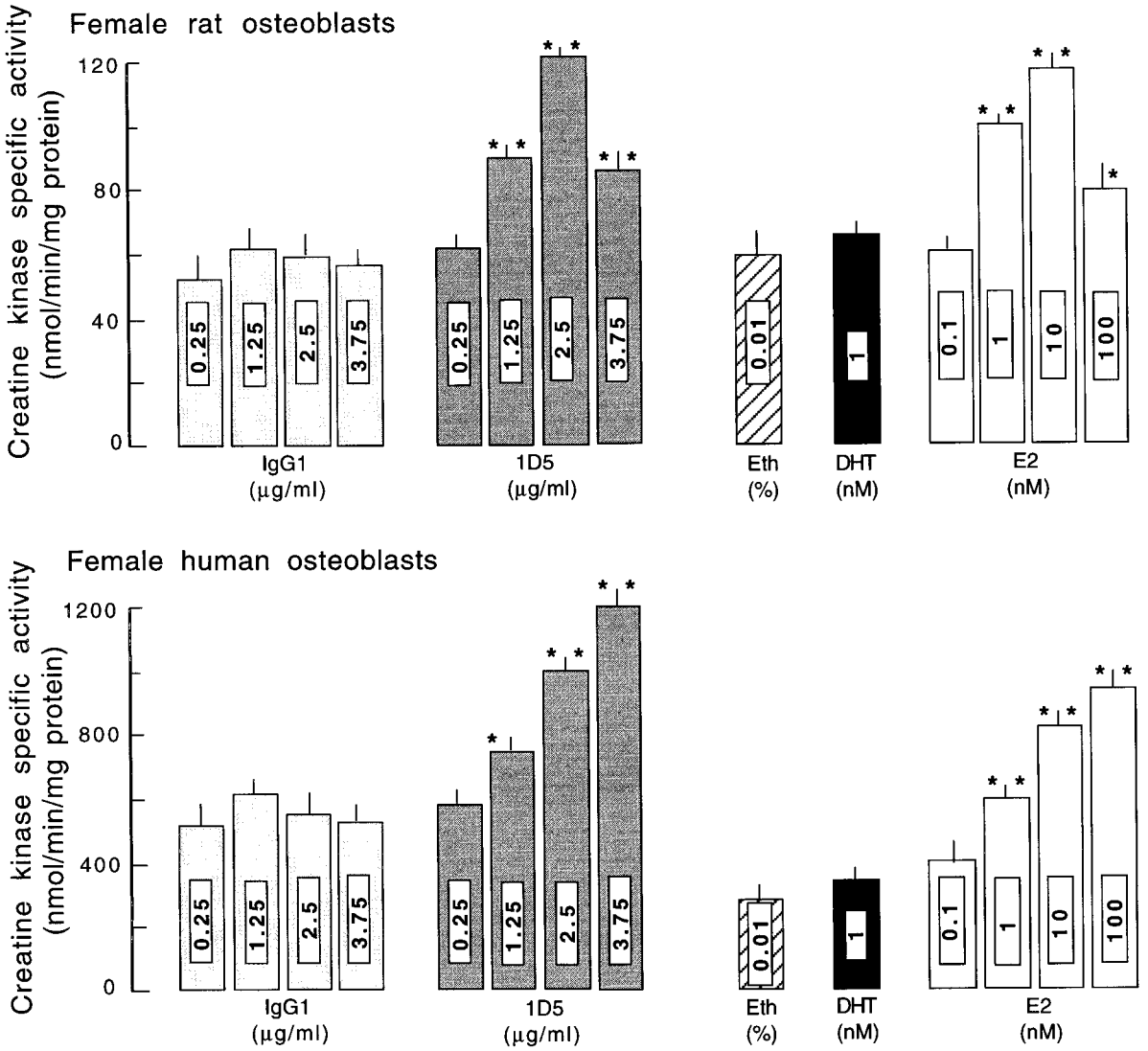

Fig. 5. Dose-dependent effects of $1D_5$ and 17β estradiol on creatine kinase specific activity in confluent female human and rat osteoblasts. The basal levels of creatine kinase activity were: 40 ± 5 nmol/min/mg protein and 390 ± 30 nmol/min/mg protein in female rat osteoblasts and in female human osteoblasts, respectively. Cells were incubated for 4 h with either various concentrations of $1D_5$, mouse IgG1, the control of $1D_5$, 17β estradiol, 5α-dihydrotestosterone or 0.01% ethanol, the vehicle of E_2 and DHT. Values are the means ± S.E.M., n = 6 and significantly different from their respective controls $^*P < 0.01$ and $^{**}P < 0.001$. Abbreviations used: IgG1 = mouse IgG1; E_2 = 17β estradiol; DHT = 5α-dihydrotestosterone.

The effects of both $1D_5$ and E_2 on CK activity were blocked by either 1 μM Tamoxifen, 1 μM Raloxifene which increased CK activity when alone, or 2 μg/ml $F(ab')_2$ in both cell types (Fig. 7).

DISCUSSION

The present report provides evidence that female human bone cells as well as female rat osteoblasts respond to the estrogen mimetic clone $1D_5$ or to 17β estradiol with a rapid increase (within 5 s) in Ca^{2+} mobilization, indicating that clone $1D_5$ can mimic the nongenomic actions of estrogens at the membrane level as well. Our results also indicate that the membrane actions of clone $1D_5$ are in part similar to that reported for estradiol [Lieberherr et al., 1993] in rat osteoblasts and involves calcium as second messenger and a Pertussis-sensitive G-protein. Moreover, $1D_5$ is also able to act at the nuclear level in causing a significant increase in creatine kinase specific activity in a gender-specific manner in female human bone cells similar to the female rat osteoblasts.

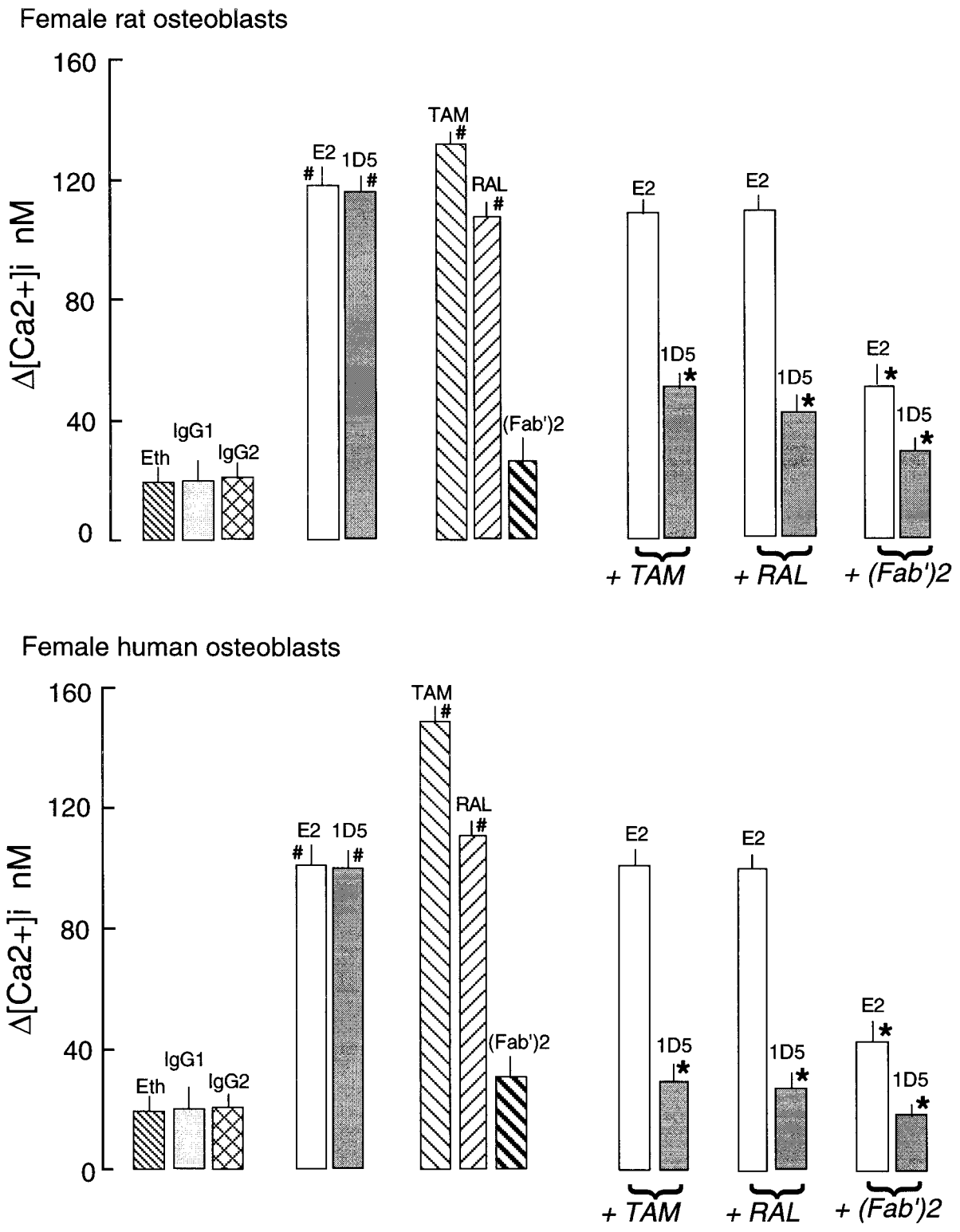

Fig. 6. Effects of Tamoxifen, Raloxifene, and (Fab′)2 on intracellular calcium response to $1D_5$ and 17β estradiol in female human and rat osteoblasts. Cells were incubated for 5 min with the antiestrogens, Tamoxifen (1 μM, TAM) or Raloxifene (1 μM, RAL) or for 2 min with 2 μg/ml (Fab′)2, an antagonist of $1D_5$, before adding 0.2 μg/ml $1D_5$ or 100 pM 17β estradiol (E_2). $[Ca^{2+}]i$ were measured at t = 10 s after the addition of either 1 μM TAM or 1 μM RAL and of either 0.2 μg/ml $1D_5$ or 100 pM E_2 in pretreated cells with the antiestrogens. Values are the means ± S.E.M., n = 6 and significantly different either from ethanol (Eth, 0.01%), mouse IgG1 (0.2 μg/ml), or IgG2 (2 μg/ml) controls, #$P < 0.001$ or 1D5 or E2 alone, *$P < 0.001$.

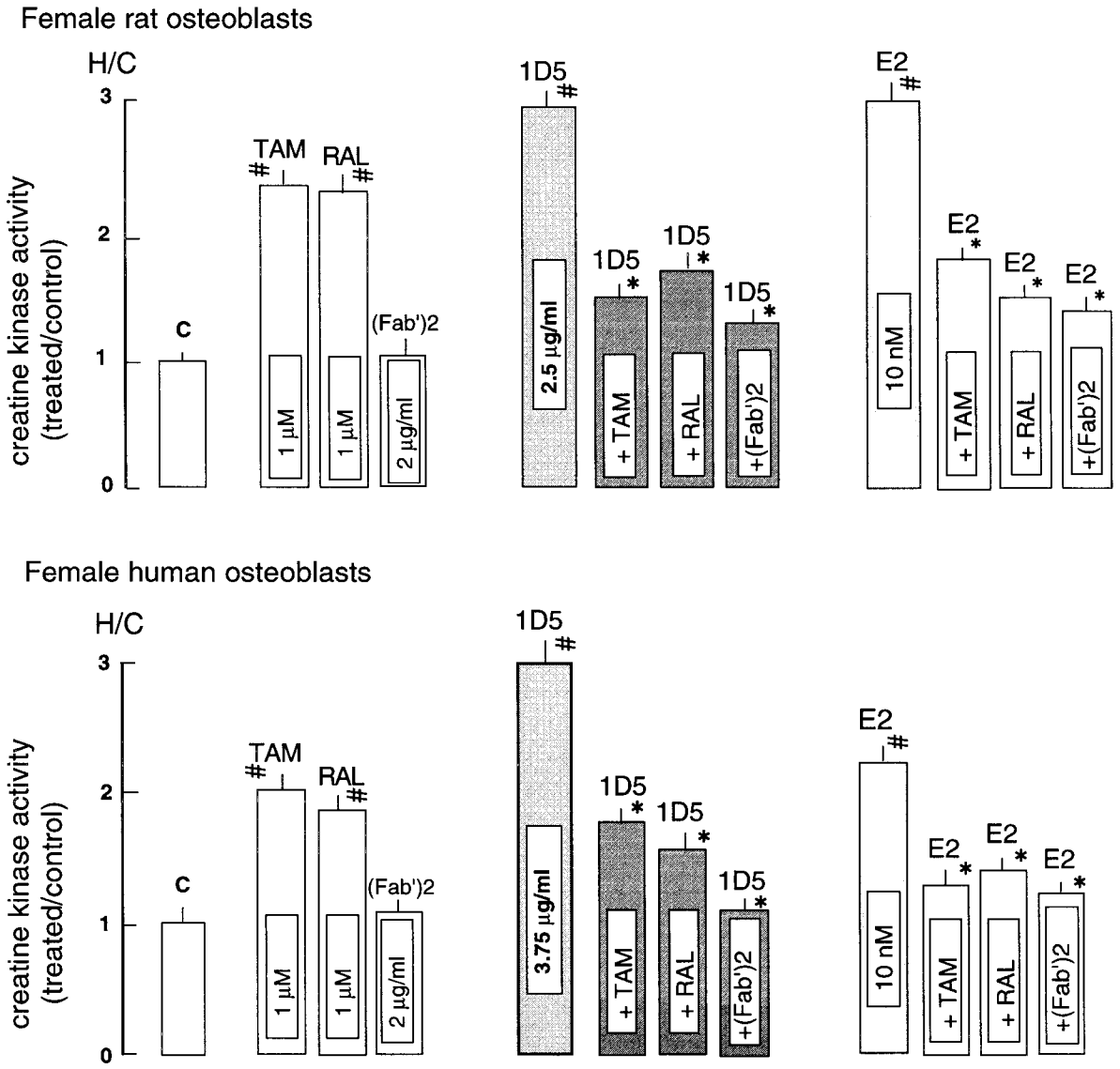

Fig. 7. Effects of Tamoxifen, Raloxifene, and (Fab')2 on creatine kinase specific activity response to $1D_5$ and 17β estradiol in female human and rat osteoblasts. Cells were incubated for 4 h simultaneously with either 2.5 µg/ml $1D_5$ for rat osteoblasts, 3.75 µg/ml $1D_5$ for human osteoblasts, 10 nM 17β estradiol (E_2) for both cell types, 1 µM Tamoxifen (TAM), 1 µM Raloxifene (RAL), or 2 µg/ml $(Fab')_2$, and with or without 1 µM TAM, 1 µM RAL or 2 µg/ml $(Fab')_2$. The basal level of CK activity was 40 ± 5 nmol/min/mg protein in rat osteoblasts and 390 ± 30 nmol/min/mg protein in human osteoblasts. Values are expressed as hormone-treated vs vehicle-treated cells and are the means ± S.E.M., n = 4 and significantly different either from controls (C), #$P < 0.001$ or $1D_5$ or E_2 alone, *$P < 0.001$.

In human and rat osteoblasts $1D_5$ or 17β estradiol triggers a transient increase in cytosolic free calcium concentration, followed by a sustained plateau phase (see Fig. 1). The dose-dependent effects in inducing a rise in $[Ca^{2+}]i$ in both cell types were bell-shaped (see Fig. 2). These results are in accordance with those previously reported for estradiol in rat osteoblasts [Lieberherr et al., 1993]. The maximal effective dose of $1D_5$ at the membrane level is 0.2 µg/ml. By contrast, at the nuclear level, both cell types require a ten fold higher concentration of $1D_5$ or estradiol to respond by an increase in CK specific activity (see Fig. 5).

Our results show that $1D_5$ rapidly (within 5 s) increases $[Ca^{2+}]i$ through two mechanisms. On the one hand, $1D_5$ like estradiol [Lieberherr et al., 1993] induces an influx of Ca^{2+} from the extracellular milieu through voltage-gated calcium channels (see Fig. 8 (mechanism 1) and

Table I). On the other hand, $1D_5$ like estradiol [Morley et al., 1992; Lieberherr et al., 1993] triggers the release of calcium from the endoplasmic reticulum through the phosphoinositide breakdown as shown by using inhibitors of phospholipase C (PLC) (see Fig. 8 (mechanism 2) and Table I). An increase in the turnover of inositol lipids in response to receptor activation is one of the major molecular mechanism used by cells for transmembrane signalling. The initial event is the hydrolysis of phosphatidylinositol 4,5 bisphosphate, a reaction catalyzed by a phosphoinositide specific PLC which generates two intracellular second messengers, 1,4,5 trisphosphate ($InsP_3$) and diacylglycerol (DAG) [Berridge and Irvine, 1989]. Ins P_3 binds to specific receptors on the endoplasmic reticulum [Supattapone et al., 1989] and mobilizes intracellular calcium whereas DAG acts as a signal transducer to increase protein kinase C activity by translocating the enzyme from the cytosol to the membrane [Nishizuka, 1989]. Thus, the elevated calcium levels induced by $1D_5$ or E_2 and, the activated PKC may initiate a cascade of protein phosphorylations that serve as the signalling pathway from the cell membrane to the nucleus, resulting in either phosphorylation of estrogen receptor itself or the phosphorylation of nuclear factors with which the receptor interacts in mediating transcription [Katzenellenbogen, 1996].

Membrane-based receptors generally transduce their signals through an interaction with the heterotrimeric guanidine nucleotide-binding proteins (G-proteins) and the activation of phosphoinositide-phospholipase C. A large number of physiological experiments have led to the postulation that there are two classes of these G-proteins: one class of G-protein-mediated responses is pertussis-sensitive, and the other is insensitive [Simon et al., 1991]. Preincubation of the female rat and human osteoblasts with PTX totally abolishes the transient Ca^{2+} peak induced by $1D_5$ corresponding to the mobilization of Ca^{2+} from the endoplasmic reticulum, but without any effect on the plateau phase corresponding to the influx of Ca^{2+} (see Fig. 4). This result is similar to what was observed with E_2 [Lieberherr et al., 1993]. The toxin seems to uncouple the "membrane receptor" from its G-protein by blocking the pathway which activates the phospholipase C (see Fig. 8).

$1D_5$ like E_2 induces increases in CK specific activity in female rat and human osteoblasts as previously described for $1D_5$ and estradiol in female rat osteoblasts [Sömjen et al., 1989, 1995]. Creatine kinase is induced directly by estradiol and others steroids by a direct interaction with the nuclear receptors [Sömjen et al., 1989], but also by peptide hormones as parathyroid hormone (PTH) [Sömjen et al., 1991] via activation of second messengers such as inositol 1,4,5 trisphosphate and Ca^{2+}, which can replace PTH in the induction of CK. So, the two mechanisms are possible. The question about $1D_5$ is whether it works directly as demonstrated by direct immunofluorescence [Mor et al., 1993; Kaye et al., 1996], or by Ca^{2+} and PLC as demonstrated by the Ca^{2+} measurements, or both (see Fig. 8). This has still to be elucidated. We showed that the concentration-dependent effects of $1D_5$ or E_2 on CK activity are bell-shaped in female rat osteoblasts, but not saturable in human osteoblasts at this range of concentrations (see Fig. 5). Although we used in both cases subconfluent cells which can be stimulated for growth, and therefore the induction of CK and DNA, this difference may arise from the stage of sexual maturation, i.e., neonatal female rats and pubertal girls.

Anti-estrogens may have steroid-like or non-steroid structure and are designated to bind to the estrogen receptor to block its function [Jordan and Murphy, 1990]. The different classes of antiestrogens can inactivate the receptor by preventing it from being available for estrogen at subsequent periods or by possibly blocking the acceptor sites for further action, or both [Spelsberg et al., 1988; Yang et al., 1996a]. Tamoxifen and Raloxifene have been developed as antiestrogens which antagonize estrogen actions in reproductive tissues by inhibiting estradiol-induced activation of estrogen responsive element (ERE)-containing genes to various extents [Jordan and Murphy, 1990; Yang et al., 1996b]. In contrast to their antagonistic activities in uterus and breast, Tamoxifen and Raloxifene function as estrogen agonists to maintain bone mass [Love et al., 1992; Black et al., 1994]. In our experimental conditions, Tamoxifen and Raloxifene are agonistic alone in increasing $[Ca^{2+}]i$ (see Fig. 6), but they have no antagonist effect in the presence of E_2 whatever the preincubation time (5 min or 4 h) with the antiestrogens. This suggests that the membrane effect of E_2 does not necessary need the binding of the ligand to the nuclear receptor. However, when cells are preincubated with Tamoxifen or

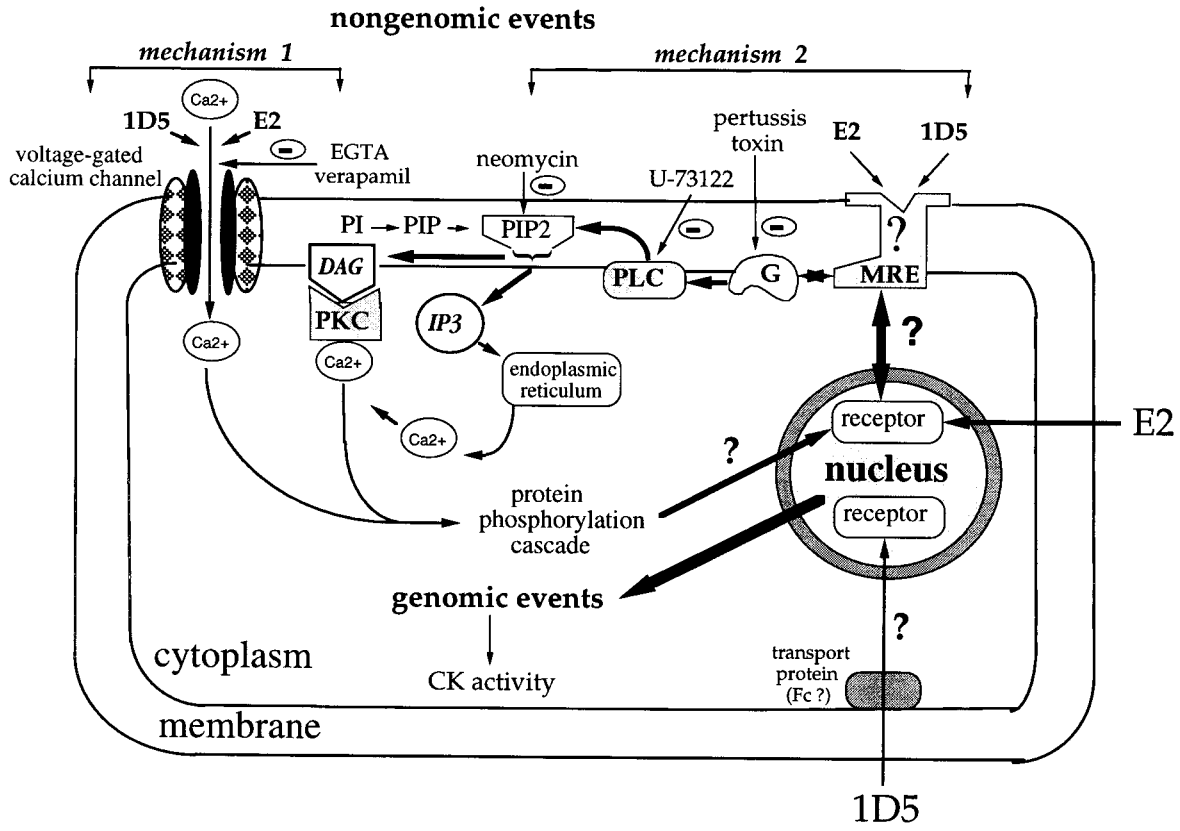

Fig. 8. Signals generated by $1D_5$ or estradiol transmitted to the nucleus in parallel pathways. MRE is a hypothetical membrane recognition element (membrane estrogen-binding sites) for $1D_5$ or E_2. The rapid increase in intracellular calcium concentration (nongenomic events) induced by $1D_5$ and E_2 involves two mechanisms: (i) an influx of Ca^{2+} from the extracellular milieu (mechanism 1) which is blocked by EGTA and verapamil; (ii) a mobilization of Ca^{2+} from the endoplasmic reticulum (mechanism 2) which is blocked by phospholipase C inhibitors (neomycin and U-73-122) and Pertussis toxin. In analogy with other membrane receptors, a G-protein-sensitive to Pertussis toxin is shown to link this MRE to phospholipase C (PLC). Activation of PKC by both Ca^{2+} and DAG may lead to a cascade of protein phosphorylations that serve as transducers from the cell membrane to the nucleus. For the genomic events, E_2 and $1D_5$ bind to the nuclear estrogen receptors, resulting in the induction of CK activity. There may be a transport protein for $1D_5$ which takes it to the nucleus, this transport protein may be the Fc transporter (?). The symbol ⊖ indicates where inhibition of the signal by various inhibitors takes place. The abbreviations are: $InsP_3$, inositol 1,4,5 trisphosphate; DAG, diacylglycerol; PKC protein kinase C; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5 bisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate.

Raloxifene, part of the $[Ca^{2+}]i$ increase induced by $1D_5$ is inhibited (see Fig. 6). In contrast, $(Fab')_2$ dimer, a proteolytic fragment of $1D_5$ with antagonist property obtained by removing the Fc fragment of $1D_5$ [Sömjen et al., 1995], blocks by about 50% the $[Ca^{2+}]i$ increase induced by either E_2 or $1D_5$ (see Fig. 6). On the other hand, Tamoxifen and Raloxifene alone act as agonists in inducing CK, but as antagonists in the presence of either estradiol or $1D_5$ (see Fig. 7) as was previously reported for Tamoxifen and $(Fab')_2$ of $1D_5$ [Sömjen et al., 1995]. This difference between the genomic and the non-genomic responses to antiestrogens may help in understanding the mechanisms involved in estradiol action, and probably indicates different interactions with the different receptors.

The membrane effects of $1D_5$ are also gender-specific as shown for estradiol [Lieberherr et al., 1993] and for the genomic effects of $1D_5$ [Sömjen et al., 1995]. Like estradiol [Lieberherr et al., 1993], $1D_5$ is not able to increase $[Ca^{2+}]i$ [unpublished data] as well as CK activity [Sömjen et al., 1995] in male bone cells. Moreover, testosterone and 5α-dihydrotestosterone had no effect on $[Ca^{2+}]i$ and CK activity in female rat and human osteoblasts (see Fig. 6) although these androgens affect intracellular calcium [Lieberherr and Grosse, 1994] and CK specific activity [Sömjen et al., 1995] in male osteoblasts.

In conclusion, the anti-idiotypic antibody $1D_5$ directed against the steroid binding site of the intracellular estrogen receptor has the capacity to mimic various activities of 17β estradiol at the membrane level as well as those at the nuclear level. This suggests that $1D_5$ may interact with membrane binding sites recognizing also estradiol (see Fig. 8), penetrate the cell as shown by immunofluorescence studies in different cell types [Amir-Zalsman et al., 1993; Mor et al., 1993] including osteoblasts [Kaye et al., 1996] and reach the nuclear receptor by an as yet uncharacterized mechanism. However, the membrane estrogen-binding proteins may be involved in the transport of the anti-idiotypic antibody to the nucleus where it activates the genome, resulting in the induction of CK activity (see Fig. 8). Our results also suggest that the steroid binding site of the membrane estrogen receptor shares some characteristics of the steroid binding site of the classic intracellular estrogen receptor since the anti-idiotypic antibody recognizes estrogen binding sites in cryostat sections prepared from human estrogen sensitive tissues [Mor et al., 1992]. Finally, this anti-idiotypic antibody can serve as a tool in demonstrating the presence of unconventional membrane receptors for estradiol. Studies of the pathways used in signal transduction and regulation of such a receptor might be important in elucidating the mechanism of action of estrogens both in normal and pathological conditions.

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