Pergamon

0960-0760(93)E0034-5

J. Steroid Biochem. Molec. Biol. Vol. 48, No. 4, pp. 325–336, 1994

0960-0760/94 $6.00 + 0.00

Estradiol-induced Down-regulation of Estrogen Receptor. Effect of Various Modulators of Protein Synthesis and Expression

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Incubation of MCF-7 cells with estradiol (E_2) down-regulates estrogen receptor (ER) resulting in a progressive reduction of the capacity of cells to concentrate selectively [^{3}H]E_2. Scatchard plot analysis failed to detect any transformation of residual receptors into peptides of lower binding affinity. [^{3}H]Estrone gave an identical ER disappearance pattern with an ER half-life comprised between 2 and 3 h. A similar value was established by incubating the cells with [^{3}H]tamoxifen-aziridine ([^{3}H]TAZ) for 1 h before the addition of excessive unlabeled E_2 which induced ER-down regulation and impeded any further labeling of the residual receptors. Submission of the [^{3}H]TAZ labeled cell extracts to SDS–PAGE revealed no progressive emergence of low molecular weight cleavage products of the receptor (<67 kDa). Two inhibitors of protein kinases, H-7 at 40 μM and H-89 at 20 μM, failed to block the E_2-induced ER down-regulation. On the contrary, the protein phosphatases 1 and 2A inhibitor, okadaic acid, was effective with concentrations higher than 0.1 μM indicating that a dephosphorylation mechanism was involved in this phenomenon. Cycloheximide (CHX) also significantly reduced the receptor decrease at concentrations higher than 1μM. G-C specific intercalating agents [actinomycin D (AMD) and chromomycin A_3 at 1 μM] also prevented ER disappearance; ethidium bromide (EB) and quinacrine were ineffective. AMD and CHX operated immediately after their addition to the medium indicating an inhibitory action on the synthesis of an RNA and/or a peptide with high turnover rate involved in ER decline. Moreover, AMD produced its suppressive effects under conditions impeding any labeling of newly synthetized receptors (i.e. [^{3}H]TAZ with an excess of unlabeled E_2) rejecting the possibility of an increasing ER production which may partially hamper its disappearance. Finally, E_2-induced ER mRNA down-regulation was similarly abolished by AMD while EB and CHX were devoid of effect.

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INTRODUCTION

The primary determinant of estrogenic effects in eukaryotic cells is the estrogen receptor (ER), a member of a large family of highly specialized transcription factors including receptors for all steroid and thyroid hormones [1, 2]. In response to estradiol (E_2) binding, ER associates strongly with enhancer-like estrogen responsive elements (ERE) located near or within responsive genes [3, 4] as well as with other components of the transcriptional machinery to trigger specific genomic responses [5].

ER does not function as a static modulator within the cell, rather its concentration is substantially modified by a number of factors including cell density [6], growth rate [7], tumor promoters [8], differentiation-inducing agents [9], cytokines [10], as well as hormones and antagonists [11]. Among the most intensively studied negative modulators of ER levels are the estrogens themselves. This phenomenon of homologous down-regulation also called "processing", has been documented in various experimental systems [12, 13]. Its finding for other steroid hormone receptors [14, 15] led to the concept that such a ligand-induced down-regulation represents a feed-back mechanism to limit the duration of hormone action on the cell.

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Received 1 June 1993; accepted 10 Nov. 1993.

MCF-7 mammary tumor cells have been widely used to study the mechanism of estrogen-induced ER down-regulation. First reports suggested its dependence on transcription since actinomycin D (AMD) and chromomycin A_3, G-C specific DNA intercalators and transcription inhibitors, were found to totally block the phenomenon while non G-C intercalators appeared to be non-effective [16]. Subsequent investigations revealed a prolonged reduction of the ER mRNA expression under E_2 treatment [17–19], which appeared not to be dependent on new protein synthesis [20] nor to an arrest of ER gene transcription in view of the fact that ER mRNA suppression was accompanied by an increase of its transcription after an early transient decrease [17, 21]. In contrast to this decline in ER mRNA expression, the loss of ER binding capacity was shown to be blocked by cycloheximide (CHX) [13]. This effect described as yet solely in uterine cells has also been observed for androgen [22] and thyroid hormone [23] receptors.

In the present study conducted on MCF-7 cells, we further explored the mechanism of ER down-regulation. Effects of E_2 and estrone (E_1) on the kinetics of ER processing were analyzed in order to evaluate the influence of the binding affinity of the ligand on this phenomenon. Effects of various inhibitors of transcription, protein synthesis and phosphorylation/dephosphorylation were also investigated.

MATERIALS AND METHODS

Compounds, antibodies

[^{3}H]E_2 ($\pm$86 Ci/mmol), [^{3}H]E_1 ($\pm$82 Ci/mmol) and [^{3}H]tamoxifen aziridine ([^{3}H]TAZ) ($\pm$20 Ci/mmol) were purchased from Amersham (U.K.). Unlabeled E_2, E_1, CHX, ethidium bromide (EB) and qinacrine were obtained from Sigma (St Louis, MO). H-7 and H-89 were purchased from Calbiochem (La Jolla, CA), AMD, chromomycin A_3 and okadaic acid from Boehringer (Mannheim, Germany). H-222 rat anti-ER monoclonal antibody was provided by Dr C. Nolan (Abbott Lab., North Chicago, IL); anti-rat IgG agarose was purchased from Sigma.

Probes

The 1300 bp *Eco*RI fragment of pOR3 (ATCC) was used as ERmRNA probe. The 36B4 specific probe was the 700 bp *Pst*I fragment of the 36B4 cDNA [24]. These fragments were introduced respectively in the pGEM3Z (Promega; Madison, WI) and pSPT18 (Pharmacia; Uppsala, Sweden) plasmids for the synthesis of anti-sense riboprobes with the Promega SP6/T7 transcription kit, using [α^{32}P]CTP (NEN-Dupont; Boston, MA).

Culture materials

Earle's based minimal essential medium (MEM) with and without phenol red, fetal calf serum (FCS), L-glutamine, penicillin, streptomycin and gentamycin were purchased from Gibco (Gent, Belgium). T-75, T-175 flasks and 6-well dishes were from Falcon (Becton Dickinson, Gent).

Culture conditions

MCF-7 cells were obtained in 1977 from the Michigan Cancer Foundation, Detroit. Since their introduction in our laboratory they were maintained at 37°C as monolayer cultures in closed plastic flasks containing MEM with phenol red supplemented with 10% heat inactivated FCS (56°C, 1 h), L-glutamine, penicillin, streptomycin and gentamycin at the usual concentrations.

ER whole cell assay: E_2-induced ER processing

MCF-7 cells maintained in monolayer culture were removed by trypsinization. Detached cells were then suspended in MEM containing 10% DCC-treated FCS [25] and plated in 6-well dishes ($\pm 1.5 \times 10^5$ cells in 2 ml per well). After 4 days of culture at 37°C in a humidified 95% air 5% CO_2 atmosphere, media were removed and cells washed gently with phosphate saline buffer (PBS).

To quantify the extent of E_2-induced ER processing, receptor concentrations were measured in the monolayer by whole cell assay [26] using phenol red-free MEM. Briefly, MCF-7 cells were incubated for different times (45 min–18 h) at 37°C with 1 ml of serum-free medium containing [^{3}H]E_2 at concentrations ranging from 0.2 to 2 nM. Additional dishes were filled with the same concentrations of [^{3}H]E_2 and a 500-fold excess of unlabeled E_2 for non-specific binding measurement. After incubation, the medium was removed and the monolayer washed three times with ice-cold saline solution. Bound labeled and unlabeled E_2 were extracted from the monolayer by a final incubation of 20 min in 1 ml ethanol at room temperature and aliquots of 200 μl of such ethanolic extracts were transferred to scintillation vials containing 3.8 ml scintillator Ecoscint H (National diagnostic, Atlanta, GE) for radioactivity counting. All measurements were performed in triplicate. Specific [^{3}H]E_2 incorporation into the cells was calculated from the difference in incorporated radioactivity after incubation in the absence or presence of an excess of unlabeled E_2 and the data were analyzed according to Scatchard [27]. In each experiment an additional 6-well dish was run in parallel for DNA measurement by the diphenylamine method [28]. Data were expressed in fmol (10^{-15} mol/μg DNA.

In some experiments a "two points" assay based on the incubation of the cells with only one concentration (1 nM) of [^{3}H]E_2 or [^{3}H]E_1 in the absence or presence of a 500-fold excess of the corresponding unlabeled hormone was employed. Data were expressed in percentage of optimal labeling, considered as the value of ^{3}H-labeled hormone specifically incorporated into the cells after 45 min of incubation [29].

SDS-gel electrophoretic characterization of ER under E_2 treatment: pulse–chase experiments

MCF-7 cells were plated in T-175 flasks in MEM containing 10% DCC-treated FCS. After 4 days of culture at 37°C in a humidified 95% air 5% CO_2 atmosphere, media were removed and cells gently washed with PBS. Cells were then incubated with 20 nM [^{3}H]TAZ for 1 h in medium containing 10% DCC-treated FCS at 37°C; for non-specific binding determinations, they were preincubated for 30 min at 37°C with 1 μM of unlabeled E_2 and maintained with unlabeled E_2 during the whole [^{3}H]TAZ labeling period. At the end of the incubation with [^{3}H]TAZ, the medium was decanted and the cells rinsed three times with 10 ml of medium containing 1 μM unlabeled E_2 to impede further labeling of residual ER (chase medium). Chase medium was then added to the culture flasks for various periods of time at 37°C (up to 18 h); zero hour controls were not incubated with this medium after labeling. At the end of incubation, medium was either discarded or collected for ER assays (see below); cells were then detached from the flask with 1 mM EDTA in Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++} and harvested by 10 min centrifugation at 300*g*. Cell pellets were washed twice with HBSS and extracted by freezing at −70°C in 0.5 ml of a buffered high salt solution (500 mM KCl in 10 mM phosphate buffer, 1.5 mM EDTA and 10 mM thioglycerol pH 7.4) containing 1 μM E_2 to avoid any further labeling by unremoved [^{3}H]TAZ. After thawing, cell extracts were homogenized with this buffer in a Teflon–glass Potter and centrifuged at 100,000*g* for 1 h. Supernatants were then submitted to a DCC treatment to remove unbound ligands, dimethylformamide was added to give a 7% final concentration and incubated with H-222 anti-ER (1 μl/ml) for 2 h at 0–4°C. Immune complexes were then adsorbed overnight on anti-rat IgG agarose, centrifuged and resolubilized in a 200 μl lysis buffer [4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% mercaptoethanol, 0.05% bromophenol blue in 500 mM Tris–HCl, pH 6.8] and their radioactivity measured by liquid scintillation in a 20 μl aliquot. For ER polymorphism analysis, 35 μl of the solubilized material was analyzed by electrophoresis in a 11% polyacrylamide gel containing 0.1% SDS, 25 mM Tris–HCl, 192 mM glycine, pH 9.2 (SDS–PAGE) [30]. Gels were stained with Coomassie brilliant blue, destained and dried after treatment with EN^{3}HANCE (NEN-Dupont) and finally submitted to fluorography to reveal [^{3}H]TAZ labeled ER bands. Bands were visualized on Kodak X-O Mat film after 2 weeks exposure at −80°C. Molecular weight (M_r) of corresponding ER forms was estimated on a computer-assisted gel scanning densitometer (Hoefer G.S. 300) with protein markers from Pharmacia (M_r 14–94 kDa).

ER contents of [^{3}H]TAZ labeled cell extracts and media were measured by Abbott enzyme immunoassay (EIA) according to the manufacturer's instructions. Before assay, media were treated with DCC to remove unlabeled hormones and concentrated by means of centriprep-10 concentrator (Amicon; Beverly, MA). Such concentrated media were also submitted to immunoprecipitation with H222, SDS–PAGE and fluorography as described above.

Quantification of ER mRNA expression

MCF-7 cells were plated in T-175 flasks in phenol red-free MEM containing 10% DCC-treated FCS. After 4 days of culture at 37°C in a humidified 95% air 5% CO_2 atmosphere, media were removed and cells washed twice with PBS. Cells were then incubated for various periods of time at 37°C in a serum-free medium in presence of a given compound aimed to block ER down-regulation (i.e. AMD or CHX). After removal of the medium, cells were rinsed twice with PBS, scraped with a rubber policeman and harvested with 10 ml PBS in a 15 ml conical tube. Cells were finally pelleted at 300*g* for 10 min and immediately frozen at −180°C until mRNA extraction.

Total RNAs were extracted by RNAzol according to the instructions of the manufacturer (Cina/Biotecx; Houston, TX). The RNAs, dissolved in RNase free water were quantified by spectrophotometry at 260–280 nm and their concentration as well as quality checked by electrophoresis through a 1% formaldehyde-agarose gel. Aliquots of 20 μg were precipitated with absolute ethanol and kept at −80°C.

Total RNA and 5 μl of the Gibco-BRL 9–0.24 kb molecular size markers were submitted to electrophoresis through a 1% agarose formaldehyde gel [31] and transferred with 10 × SCC to GeneScreen Plus membrane (NEN-Dupont) for 2 h with a Vacugene 2016 aparatus (LKB-Pharmacia). The membranes were treated according to the manufacturer's instructions. The blots were hybridized simultaneously with 7.5×10^6 cpm of the riboprobes according to the conditions described by Melton *et al.* [32], except that 1% SDS was included in the prehybridization and hybridization buffers. Hybridizations were carried out at 55°C in a hybridization oven. The blots were washed five times in 0.1% × SCC, 0.1% SDS at 65°C and autoradiographed with regular intensifying screens at −70°C. The 36B4 specific probe was used to correct for the differences in the actual amounts of RNA loaded on the gel. The autoradiographic signals were quantified by Ultrogen Scan (LKB-Pharmacia).

RESULTS

Effects of E_2 on turnover rate, molecular weight and disappearance of ER

MCF-7 cells were incubated at 37°C with [^{3}H]E_2 at concentrations ranging from 0.2 to 2 nM for various times (45 min–18 h) and their ER content determined by whole cell assay. Data analyzed according to Scatchard revealed a time-dependent decrease

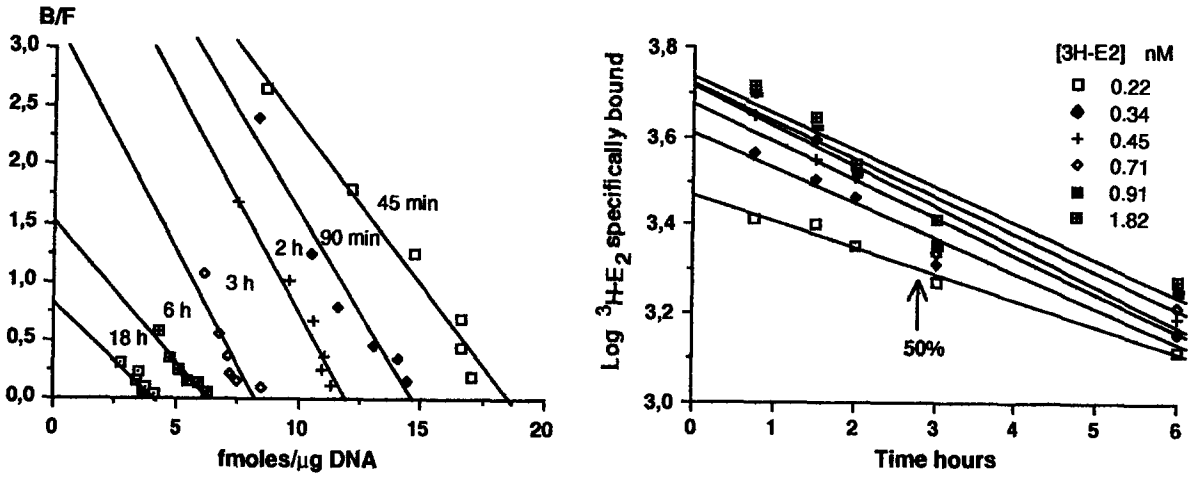

Fig. 1. E_2-induced processing. MCF-7 cells were incubated for various times with increasing concentrations of $[^3H]E_2$ in the presence or absence of an excess of unlabeled E_2. ER levels were subsequently measured by whole cell assay. The figure on the left referring to a Scatchard plot analysis of the binding data shows a time-dependent loss of ER binding capacity without any significant loss of binding affinity of the unprocessed receptors (K_d range = 0.41–0.87 × 10^{-10} M). Kinetic analysis of the data (right) reveals an ER half-life of about 3 h independently of the $[^3H]E_2$ concentration used.

of $[^3H]E_2$ binding capacity without any significant change of the dissociation constant of the binding reaction (almost parallel straight lines, K_d range = 0.41–0.87 × 10^{-10} M) indicating that ER processing occurred without any modification of binding affinity for E_2 of the residual (unprocessed) receptors (Fig. 1 left panel). Kinetic analysis of the data (Fig. 1, right panel) gave an ER half-life of about 3 h whatever the $[^3H]E_2$ concentration used. Experiments carried out with only one saturating concentration of $[^3H]E_2$ (1 nM) confirmed the maintenance of approx. 50% of the maximal original binding capacity after 3 h of incubation and revealed a new steady state of ER corresponding to 25% of the maximal binding capacity after 6 h of incubation (Fig. 2). A similar time-course of ER processing was obtained when $[^3H]E_1$ was used as ligand instead of $[^3H]E_2$, except that the new steady state still reached ≈45% of the maximal binding (Fig. 2) because of the lower processing capacity of this ligand.

Interestingly, a same kinetic of ER disppearance was observed when $[^3H]E_2$ was removed from the medium

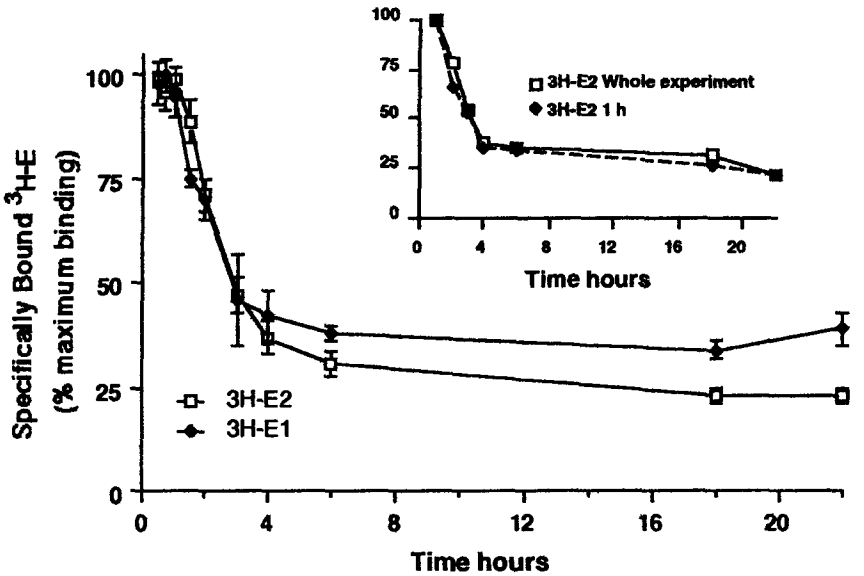

Fig. 2. Kinetics of estrogen-induced ER processing. MCF-7 cells were incubated for indicated times with 1 nM of either $[^3H]E_2$ or $[^3H]E_1$ in the presence or absence of an excess of the corresponding unlabeled hormone. ER levels were subsequently measured by whole cell assay with both ligands. The figures expressed as the mean ± SEM of several experiments revealed a 50% decrease of specific $[^3H]E_2$ or $[^3H]E_1$ binding capacity after about 3 h incubation. ER levels after prolonged incubation (new steady state) were lower with $[^3H]E_2$ than $[^3H]E_1$. Removal of the hormone after the first hour of incubation did not alter the kinetics of ER processing (inset).

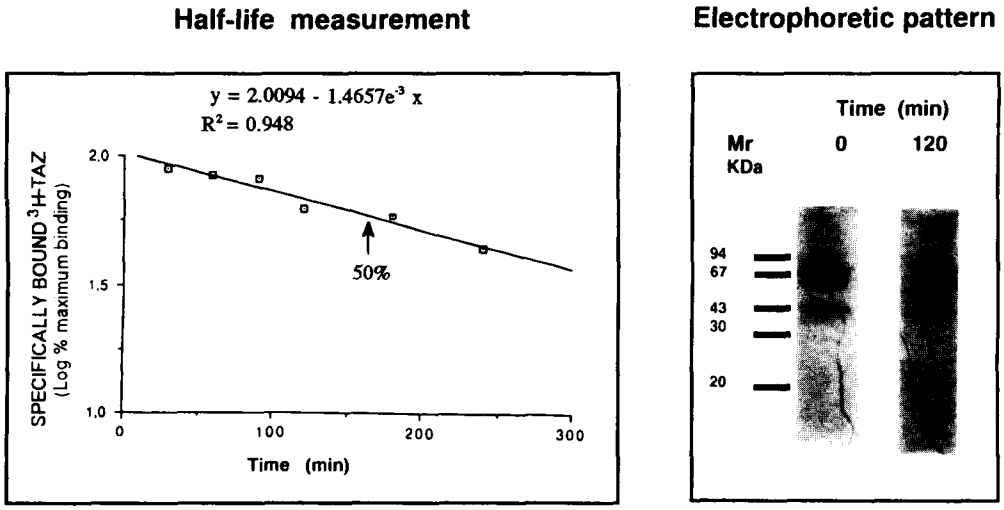

Fig. 3. Electrophoretic properties of ER covalently labeled with [^{3}H]TAZ. MCF-7 cells were labeled with [^{3}H]TAZ for 1 h and subsequently exposed to 1 μM unlabeled E_2 (up to 4 h). After extraction with 0.5 M KCl, ER contents were selectively immunoabsorbed with H222 anti-ER monoclonal antibody. Radioactivity of a part of the extracts were measured to evaluate ER half-life (data expressed as percent of control value); kinetic analysis of the data gave a value of about 3 h (left panel). The other part of extracts were submitted to SDS–PAGE to investigate the influence of processing on ER polymorphism (right panel). Control [^{3}H]TAZ labeled cells revealed the presence of native ER (67 kDa) associated with a few degradation products of lower molecular weight; by 2 h of E_2 treatment there was a significant reduction in the amounts of all ER forms without any change in ER polymorphism.

after the first hour of incubation (period for optimal labeling) and the cells gently washed. Under such conditions all [^{3}H]E_2 extracted from the cells corresponded to specifically bound steroid since non-specific binding was practically negligible. Hence, ER processing appears to be the consequence of its activation and once accomplished, presence of the hormone is no longer necessary (Fig. 2, inset).

Cells labeled with [^{3}H]TAZ for 1 h and subsequently exposed to 1 μM E_2 gave a similar ER half-life of $\approx$3 h after E_2 addition (Fig. 3, left panel). Hence, [^{3}H]TAZ labeling of the cells did not impede the E_2-induced processing as confirmed by assessing ER disappearance by enzyme immunoassay (Fig. 4). Of note, ER disappearance was not observed when E_2 was not added to the medium indicating that [^{3}H]TAZ was unable to induce ER processing at the concentration used for the labeling of the receptor (Fig. 4). Analysis of extracts from such [^{3}H]TAZ labeled cells by SDS–PAGE revealed the presence of native ER (67 kDa) associated with a few degradation products of lower molecular weight. After 2 h of E_2 treatment, there was a noticeable disappearance of all ER forms without any significant relative increase in the amounts of the degradation products (Fig. 3, right panel). Computer assisted analysis of the ER band patterns confirmed this statement indicating that processing did not modify the receptor molecular polymorphism. This paradoxal absence of increase of cleavage products, already reported in uterine cells [13], raised the question of the location of processed receptors. In view of the fact that E_2 treatment increases the secretory activity of MCF-7 cells [33, 34], we explored the possibility of extracellular release of ER. On media from cells submitted to E_2 treatment (1 nM) or pulse–chase experiments of [^{3}H]TAZ labeled cells we failed to detect any ER by both EIA and SDS–PAGE/fluorography (data not shown).

Effect of AMD on E_2-induced processing

AMD is known to reduce the E_2-induced decrease of [^{3}H]E_2 binding capacity in MCF-7 cells [16, 35]. Confirming this observation, we found that the time-dependent loss in binding capacity produced by 1 nM

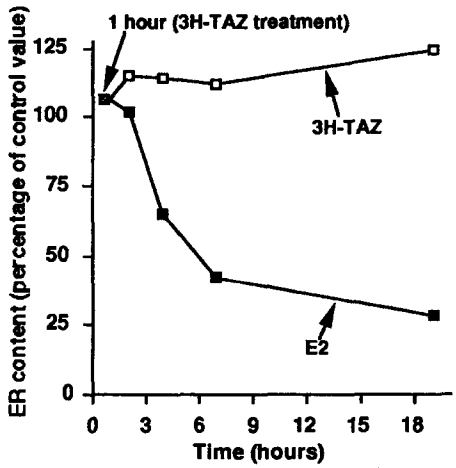

Fig. 4. E_2-induced ER processing on cells pretreated with [^{3}H]TAZ. MCF-7 cells were incubated for 1 h with 20 nM [^{3}H]TAZ, medium was then discarded and replaced by fresh medium with either 20 nM [^{3}H]TAZ or 1 μM E_2 for various times. Harvested cells were homogenized and their ER content determined by using Abbott ER EIA. Results, presented as percentage of control value (100%), reveal that ER down-regulation was only observed after E_2 addition.

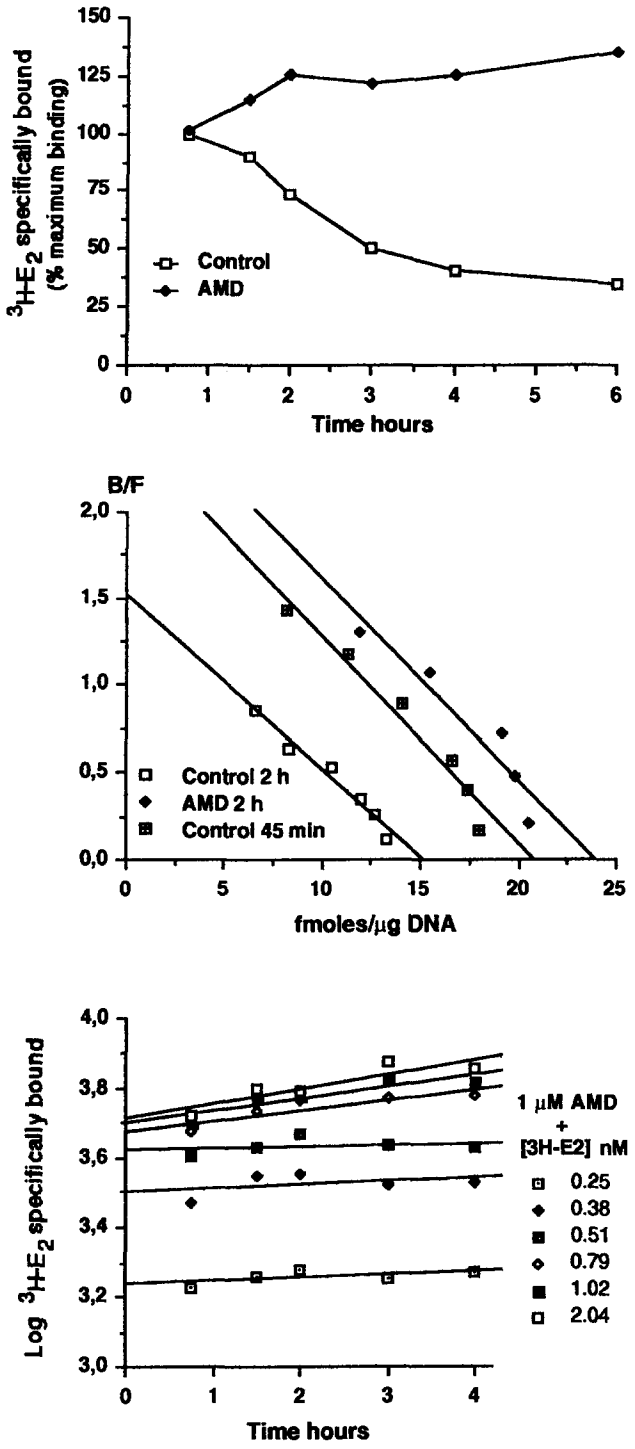

Fig. 5. Effect of AMD on E_2-induced ER processing. MCF-7 cell cultures to which 1 μM AMD was or was not added were incubated for indicated times with 1 nM of [^{3}H]E_2 in the presence or absence of an excess of unlabeled E_2. ER levels measured by whole cell assay revealed that AMD totally blocked ER disappearance producing a slight increase in [^{3}H]E_2 binding capacity (upper panel). Scatchard plot analysis of representative binding data (2 h incubation) shows that the drug operated without modification of the binding affinity of the receptor for the hormone (K_d value $\times 10^{-10}$ M: Control 45 min = 1.46; Control 2 h = 1.69; 1 μM AMD 2 h = 1.34) (middle panel). Kinetic analysis of the data gave straight line patterns with a slight positive slope (lower panel) (compare to Fig. 1, right panel).

[^{3}H]E_2 was totally abrogated by 1 μM AMD (Fig. 5, upper panel). In fact, Scatchard plot analysis of the data revealed a slight increase of binding capacity depending on both incubation time and AMD concentration (Fig. 5, middle panel and Fig. 6). Kinetic analysis of these data gave almost parallel straight lines with a slightly positive slope (Fig. 5, lower panel vs Fig. 1, right panel); identical patterns were obtained when AMD was added to cells 1 h before or after their labeling with [^{3}H]E_2 (data not shown). Values found at the time of optimal binding were not modified by the presence of the intercalating agent indicating that it does not block or delay the binding of the hormone to receptor.

In order to investigate whether the antagonistic property of AMD may be generalized to other intercalating agents, cells were treated for 1 or 3 h with 1 nM [^{3}H]E_2 in the presence of either AMD, chromomycin A_3, quinacrine or EB at 0.1 and 1 μM. Only chromomycin A_3 and AMD which intercalate at G-C base pairs on DNA were effective, AMD being the most powerful agent (Fig. 6).

Remarkably, AMD blocked the E_2-induced ER down-regulation under conditions impeding any labeling of newly synthetized receptors (i.e. [^{3}H]TAZ with an excess of unlabeled E_2; pulse–chase experiments) rejecting the possibility of an increase of ER production in its presence which would have partially hampered its disappearance. On the contrary, EB failed to block the E_2-induced ER down-regulation confirming the specificity of the AMD effect (% of maximum [^{3}H]TAZ binding after 3 h E_2 treatment: no drug = 54%; 1 μM AMD = 98%; 1 μM EB = 51%).

Effect of CHX on E_2-induced ER processing

Incubation of MCF-7 cells with 1 nM [^{3}H]E_2 in the presence of CHX revealed that this inhibitor of protein synthesis partially blocked ER processing (Fig. 7, left panel) in a concentration-dependent manner (Fig. 7, right panel). This effect occurred without any significant variation of the binding affinity of the residual ER as revealed by Scatchard plot analysis (K_d values after 3 h of E_2 treatment: Control = 1.19 $\times 10^{-10}$ M; CHX 50 μM = 1.28 $\times 10^{-10}$ M). Blockade of ER processing also occurred when CHX was added to cells after 1 h of incubation with [^{3}H]E_2 (optimal labeling time) or when the processing had already started (90 min after addition of [^{3}H]E_2). CHX at the highest concentration used (75 μM) did not significantly modify the maximal [^{3}H]E_2 binding capacity indicating that it did not impede or delay hormone binding to the receptor. Interestingly, CHX failed to block ER processing when it was added before [^{3}H]E_2 labeling (2 h of incubation followed by its removal at the time of labeling) (Fig. 7, left panel); the sole detectable effect found under such conditions was a reduction of optimal binding capacity ($\approx$35%) probably due to an inhibition of ER synthesis. This hypothesis was supported by the fact that the residual receptor levels (steady-state) measured in the absence of CHX corresponded to the extent of

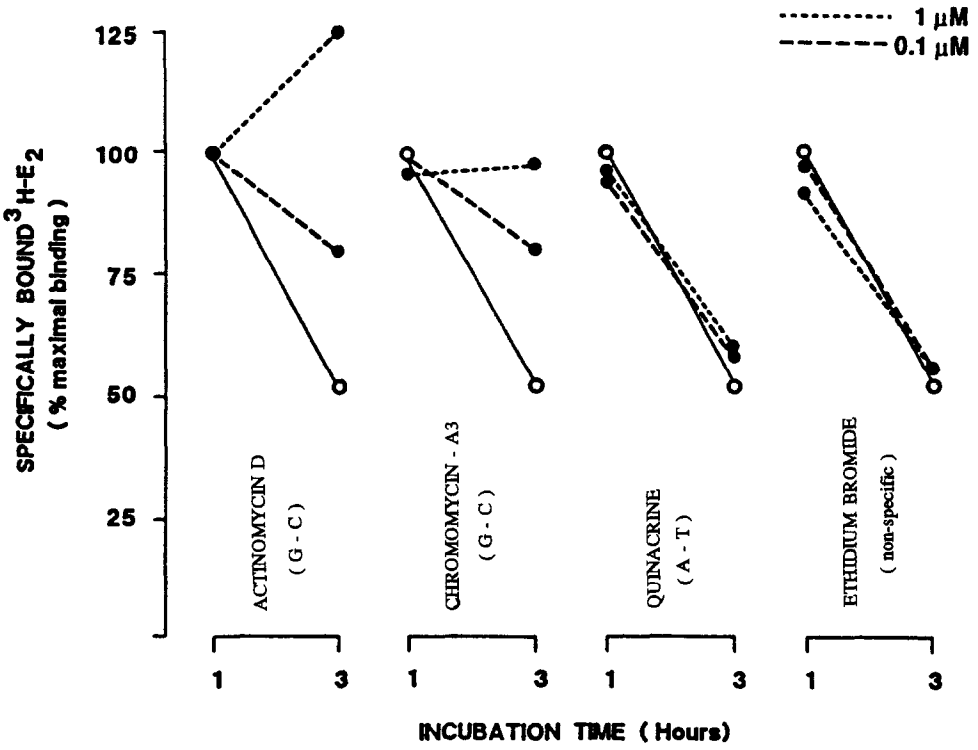

Fig. 6. Effect of different DNA intercalators on E_2-induced ER processing. MCF-7 cell cultures to which a given DNA intercalator was added at 0.1 or 1 μM were incubated for various times with 1 nM of [^{3}H]E_2 in the presence or absence of an excess of unlabeled E_2. ER levels measured by whole cell assay revealed that drugs intercalating at G-C base pairs on DNA solely impeded ER disappearance, AMD being the most powerful compound.

ER decline found when the drug was added at the time of [^{3}H]E_2 labeling.

Effect of protein kinases and phosphatases inhibitors on E_2-induced ER processing

In order to determine if a phosphorylation process is involved in E_2-induced ER processing, MCF-7 cells were incubated for 1 or 4 h with 1 nM [^{3}H]E_2 in the presence or absence of two serine/threonine protein kinases inhibitors. As shown in Fig. 8 (upper panel) H-7 at 40 μM and H-89 at 20 μM failed to affect the E_2-induced ER disappearance; a 2 h preincubation before [^{3}H]E_2 labeling was also ineffective (data not shown). Of note, while H-7 did not affect maximal

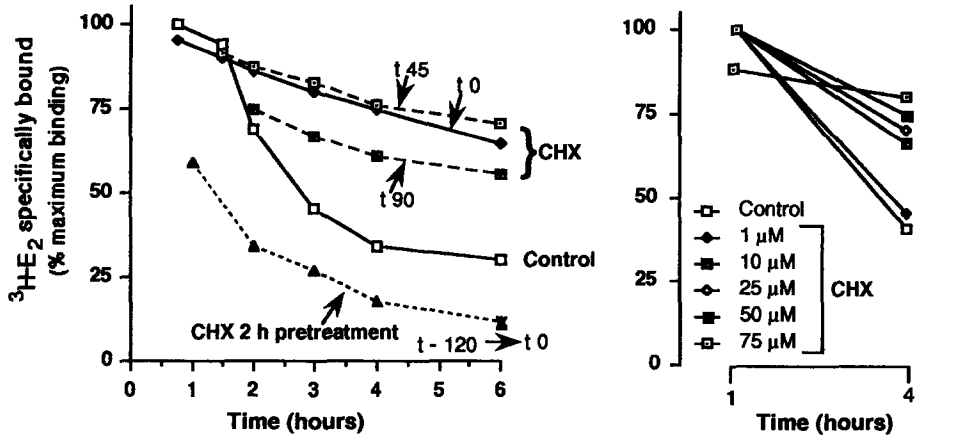

Fig. 7. Effect of CHX on E_2-induced ER processing. MCF-7 cells were incubated for indicated times with 1 nM of [^{3}H]E_2 in the presence or absence of an excess of unlabeled E_2. At various times, 50 μM CHX was added to the medium (2 h treatment prior [^{3}H]E_2 labeling: $t = 120$; at [^{3}H]E_2 labeling: $t = 0$; 45 min after [^{3}H]E_2 labeling: $t = 45$; 90 min after [^{3}H]E_2 labeling: $t = 90$). ER levels measured by whole cell assay revealed that CHX added at time of or after labeling partially blocked ER disappearance while pretreatment with the drug and posterior removal produced only a reduction of optimal binding capacity (left panel). Cells incubated as above for 1 or 4 h with various concentrations of CHX showed a concentration-dependent effect (right panel).

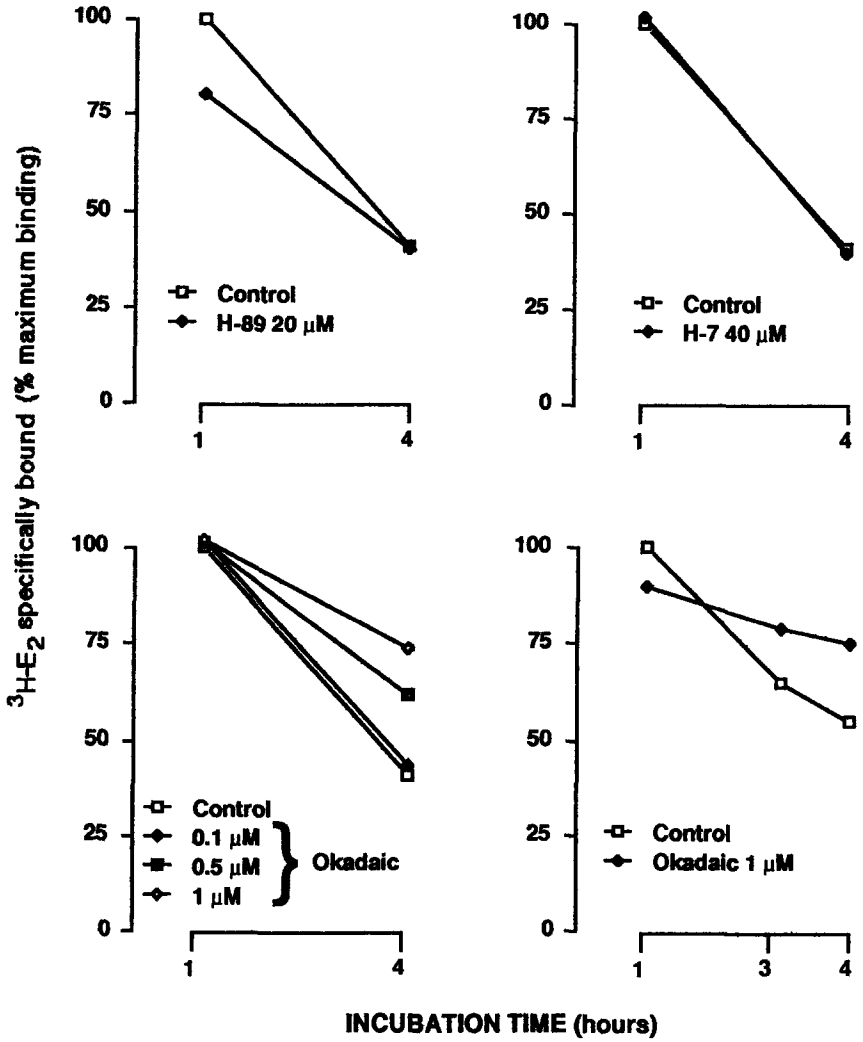

Fig. 8. Effect of protein kinases and protein phosphatase inhibitors on E_2-induced ER processing. MCF-7 cell cultures to which either H-89 (selective PKA inhibitor) at 20 μM or H-7 (broad range PK inhibitor) at 40 μM was added were incubated for 1 or 4 h with 1 nM of [^{3}H]E_2 in the presence or absence of an excess of unlabeled E_2. ER levels measured by whole cell assay revealed that H-89 and H-7 both failed to affect ER disappearance though the former produced a slight inhibition of [^{3}H]E_2 optimal binding capacity (upper panel). Okadaic acid assessed in two different experiments partially blocked the ER disappearance in a concentration-dependent manner without significantly affecting the optimal binding capacity (lower panel).

binding capacity of the cells, H-89 always produced a slight reduction ($\approx$20%) suggesting a participation of PKA [36] in the ability of newly synthetized receptors to bind E_2. On the contrary, okadaic acid, a specific inhibitor of phosphatases 1 and 2A, partially blocked ER processing in a concentration-dependent manner (concentrations higher than 0.1 μM) without significantly affecting the maximal [^{3}H]E_2 binding capacity of the cells (Fig. 8, lower panel). A dephosphorylation process of ER or of a protein regulating its turnover seems, therefore, involved in the progressive disappearance of the receptor.

Estrogen regulation of ER mRNA

The effect of 1 nM E_2 on the levels of ER mRNA in MCF-7 cells was investigated. A significant decrease of ER mRNA was rapidly observed reaching $\approx$50% of the control value after 4 h of incubation; after 18 h levels were reduced to approx. 30% of the control (Fig. 9, upper and lower panels). This E_2-induced suppression of ER mRNA was blocked by 1 μM AMD (Fig. 9, lower left panel); the decline on ER mRNA detected after 4 h in the sole presence of this agent was only 25% indicating that E_2 accelerated by 2-fold ER mRNA degradation. EB at 1 μM was ineffective in this regard confirming the specificity of action of AMD (Fig. 9, lower right panel). In contrast, CHX at 50 μM did not abolish the negative effect of E_2 on ER mRNA levels (CHX alone had no significant effect) (Fig. 9, lower right panel).

DISCUSSION

In monolayer culture of MCF-7 cells by using two methods of ER measurement namely [^{3}H]E_2 whole cell assay (a ligand exchange assay) and [^{3}H]TAZ covalent labeling before E_2 addition, we confirmed [16, 37] a ER half-life of approx. 3 h under E_2 stimulation. After

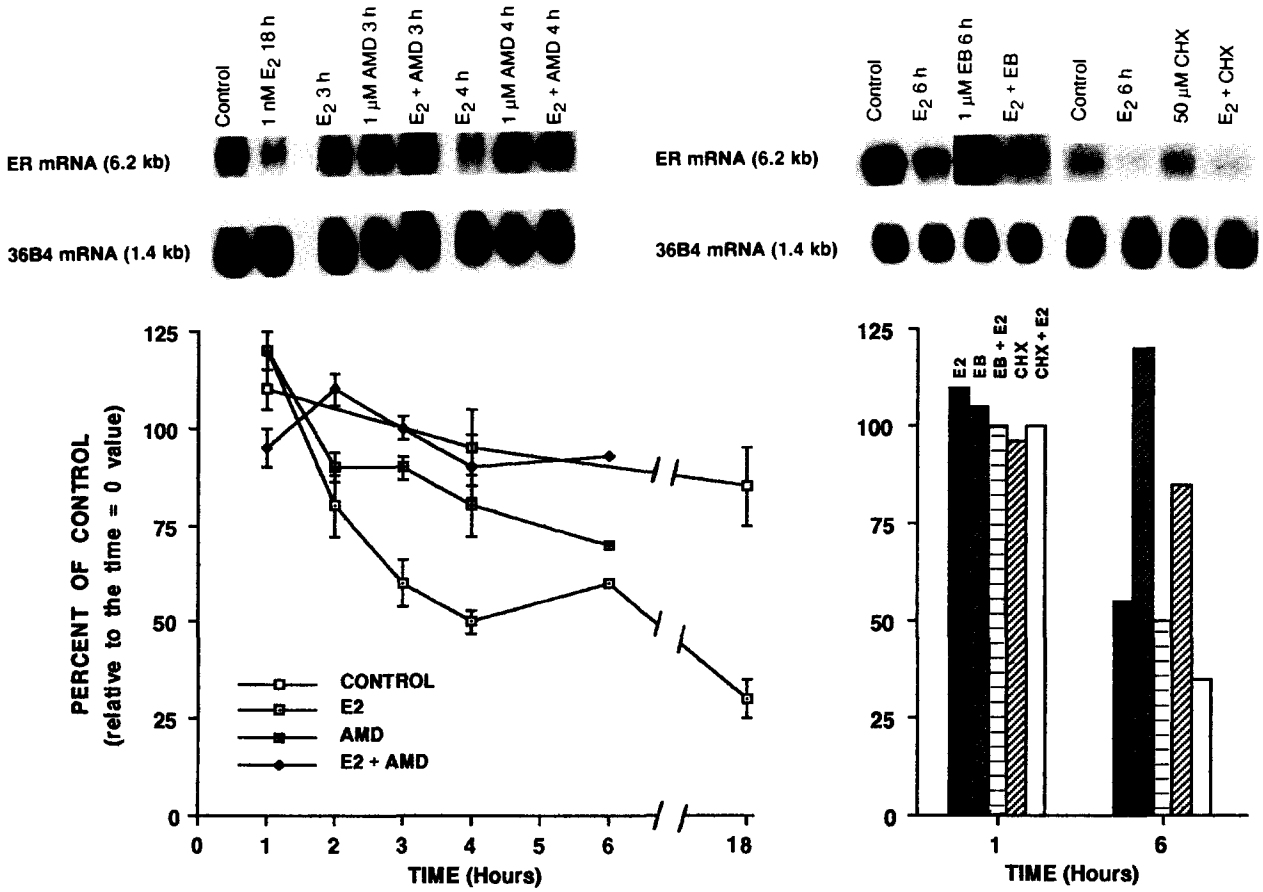

Fig. 9. E_2-induced ER mRNA down-regulation. MCF-7 cells were incubated in serum-free medium with 1 nM E_2 in the presence or absence of a given compound aimed to block ER mRNA down-regulation (i.e. AMD, EB and CHX). At indicated times cells were harvested and total RNA extracted with RNAzol. 20 μg of total cellular RNA were submitted to eletrophoresis and Northern blotting and hybridized simultaneously with 7.5×10^6 cpm of ER and 36B4 riboprobes. The blotts were autoradiographed with regular intensifying screens at -70°C for 3 days and the signals quantified by Ultrogel Scan. An autoradiograph representative of various experiments is shown (upper panel). The values were expressed as the ratio of the integrated ER signals divided by the 36B4 integrated signals. Data representing the average of two different experiments $\pm$ SEM are expressed as percent of the controls. 1 nM E_2 produced a time-dependent decrease in ER mRNA expression reaching 50% of the control value after $\approx$4 h incubation. This effect was blocked by 1 μM AMD (upper and lower left panel). EB at 1 μM and CHX at 50 μM were ineffective in this regard (upper and lower right panel).

6 h of hormone treatment a new ER steady state was reached corresponding to about 30% of the level found after the first hour (original maximum level). In addition, labeling of cells with [^{3}H]E_1 revealed no major influence of the binding affinity of the ligand on the kinetic of ER processing. During this period of ER disappearance we failed to detect any transformation of the residual receptors into peptides of lower binding affinity or a modification of ER molecular polymorphism as demonstrated by [^{3}H]TAZ labeling of the cells. Within the medium from E_2 stimulated cells we also failed to detect any ER cleavage product in a form that could be detected by [^{3}H]TAZ labeling and EIA. Therefore, the question of the location of the processed ER remains unsolved.

The E_2-induced ER loss analyzed here by ER whole cell assay, which bypass eventual difficulty of receptor extraction and further labeling, corresponds to a decrease of the receptor protein as previously shown by us and others [38, 39]. Interestingly, withdrawal of [^{3}H]E_2 after the first hour of incubation gave the same time-course of ER processing pattern indicating that this phenomenon probably results from activation of the receptor and/or subsequent binding to DNA, once the latter(s) is accomplished, the presence of the activating ligand for ER down-regulation is no longer required.

Whatever the mechanism for the E_2-induced ER down-regulation could be, it is clear that it is fully prevented by AMD and chromomycin A_3, both of which recognize G-C base pairs on DNA. Binding characteristics of ER were unaffected throughout the treatment with AMD. In fact, a slight increase in [^{3}H]E_2 binding capacity occurred in the presence of this drug indicating that ER mRNA could still be translated into new peptides; this increase, of course, was not detected under conditions impeding any labeling of newly synthetized receptors (i.e. [^{3}H]TAZ labeling

with an excess of unlabeled E_2). The effect of AMD was very rapid suggesting an inhibitory action on the synthesis of an RNA and/or an enzyme implicated in ER mRNA and peptide degradation. In this regard, it could be stressed that ER mRNA down-regulation occurring under 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) treatment is also abrogated by AMD; although the underlying mechanism is not established, the participation of a short lived RNA molecule with catalyic activity has been proposed [40]. Another proposed hypothesis is the trapping of the receptor within the chromatin matrix which would block its release from the nucleus as a receptor–RNA complex, a process postulated to be required for further RNA and subsequent protein synthesis [41].

Assessment of ER mRNA levels under E_2 treatment revealed a time-dependent decrease similar to the reduction of [^{3}H]E_2 binding capacity. Whether this phenomenon is of transcriptional or posttranscriptional nature is an unsolved question. In this regard, it has recently been shown that ER could bind to a portion of its cDNA suggesting a mechanism of autologous ER down-regulation [42]. On the other hand, in the MCF-7 model investigated here, E_2 was reported to cause an early transient drop of the transcription rate of ER mRNA; this rate subsequently rises to its original level even though ER mRNA and protein synthesis remain repressed indicating that post-transcriptional regulation is at least implicated in ER down-regulation [17, 21]. The additional observation that steroid hormones regulate the stability of some mRNAs introduced the concept of interrelationship between ER and RNA [43–45]. The fact that treatment of cytosolic ER with ribonuclease induces its interaction with DNA cellulose and matrices adsorbing activated receptors ([46] and unpublished data from our laboratory) support such an hypothesis of a functional role of ER–RNA associations.

In agreement with a preliminary report [20], we failed to show any effect of CHX on the E_2-induced ER mRNA decrease suggesting that the latter is independent of protein synthesis. In contrast, our results show that CHX arrests the ER peptide disappearance immediately after its addition. Treatment of the cells with CHX before [^{3}H]E_2 labeling did not alter the kinetics of ER processing suggesting the induction by the hormone of a rapidly turning over enzyme involved in ER degradation. This blockade of E_2-induced ER processing by CHX was accompanied by a slight decrease in the binding capacity of the cells probably due to an inhibition of newly synthetized receptors from a preexisting ER mRNA pool, as shown by a 2 h pretreatment before [^{3}H]E_2 labeling. Under such conditions a 35% inhibition of the original [^{3}H]E_2 binding capacity was observed in agreement with a more prolonged ER half-life ($\approx$5 h) already reported in the absence of estrogenic stimulation [37].

Our preliminary investigation with okadaic acid, a specific protein phosphatases 1 and 2A inhibitor [47], suggest the involvement in the E_2-induced ER processing of a dephosphorylation of the activated ER and/or a protein regulating its turnover. On the other hand, our results did not detect any implication of PKC as well as PKA on this phenomenon which contrasts with the TPA-induced ER down-regulation that has been claimed to be mediated by PKC [48]. In this regard, it should be stressed that a cross-talk between ER and Ca^{++}-dependent signal pathways through transmembrane signaling has been reported [49]. This interrelationship may represent an important modulatory mechanism of ER turnover since an increase in intracellular Ca^{++} causes a time-dependent down-regulation of ER mRNA and ER levels similar to that found with E_2 [50]. Supporting this concept is the recent observation that E_2 produces a rapid increase of intracellular Ca^{++} by activating PLCα [51–53] which displays high homology with a segment in the estrogen binding domain of ER [54, 55]. PLCα yields second messengers inositol triphosphate, diacylglycerol and arachidonic acid [56, 57] known to modulate the binding capacity of steroid receptors [58, 59].

In conclusion, estrogen-induced decrease of ER concentration is a complex mechanism depending on the production of an RNA and/or a degradative enzyme with high turnover rate. At least two mechanisms appear to be implicated in this phenomenon: first, a transcriptional and/or posttranscriptional ER mRNA regulation which could alter its synthesis and stability; second, a phosphorylation/dephosphorylation process of hormone-bound receptors [and/or related protein(s)] associated with an accelerated disappearance of ER. Variation in the intracellular Ca^{++} level could be involved in these regulatory mechanisms.

Acknowledgements—We warmly thank Dr P. Chambon for 36B4 clone. This work was supported by Fonds Lefèvre and CGER; M. Borras is a recipient of grant from Oeuvre Belge du Cancer and A. H. Al Khissiin from l'AGCD; L. Hardy and R. Gol-Winkler are recipients of a Télévie grant; R. Gol-Winkler is a Chercheur qualifié du FNRS.

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