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A One Minute Pulse of Estradiol to MCF-7 Breast Cancer Cells Changes Estrogen Receptor Binding Properties and Commits Cells to Induce Estrogenic Responses

Angela M. Otto

Lehrstuhl Pharmazeutische Chemie II, Institut für Pharmazie, Universität Regensburg, D-93040 Regensburg, Germany

Changes in estradiol (E_2)-binding parameters can be detected within minutes, while the estrogenic responses are manifested after several hours or days of continuous exposure to the steroid. The goal of this study was to determine the time of commitment for the induction of transcription-dependent responses in the human breast cancer cell line MCF-7. In cultures grown in steroid-deprived serum, a pulse of 1 nM E_2 as short as 1 min was sufficient to maximally increase the level of the progesterone receptor, as determined by binding of the progestin [^{3}H]ORG.2058 after 2 days, and to partially stimulate cell proliferation for 5 days. From uptake experiments it was calculated that after 1 min about 7000 E_2 molecules were bound per cell, enough to occupy 5% of the approx. 150,000 estrogen receptors per cell. Preincubating cells with unlabelled E_2 for 1 min lead to a loss of [^{3}H]E_2-binding capacity. As analysed by Scatchard plot, this loss was due to a decrease in the number of exchangeable binding sites and, to a lesser extent, to an increase in the dissociation constant. For up to 30 min of E_2-incubation the level of receptor protein remained constant as determined by immunoassay with the anti-ER monoclonal antibodies D547 and H222. The dissociation kinetics of [^{3}H]E_2 bound by MCF-7 cells after a 5 min pulse were biphasic, with the slower phase having a rate of 2.3×10^{-3} min. This rate is characteristic of the activated ER. The estrogenic response is thus committed by E_2 within less than 1 min and evoked by the activation of a small fraction of estrogen receptors.

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INTRODUCTION

The binding of estrogen to its receptor is a prerequisite for stimulating different types of specific responses in the target cell. The results of numerous studies on the biochemistry and intracellular localization of the estrogen receptor (ER) have culminated in a generally accepted model [1–4]. It proposed that the cytoplasmic unoccupied receptor is associated with other proteins to give it its characteristic 8S sedimentation coefficient. Upon binding of estradiol (E_2), the receptor is "transformed" (or activated): this state describes biochemical changes including a reduction in the sedimentation coefficient to 5S due to disaggregation of the associated proteins and receptor dimerization, high affinity binding of the ligand, and translocation into the nucleus, and strong DNA binding. There is consensus in that the ER binds to specific DNA sequences (estrogen responsive element) on the inducible genes [5]. There is also evidence that both unoccupied and occupied receptors are located in the nucleus [6–8], but the significance is still a matter of debate.

The biochemical binding parameters of the ER have been studied mainly in cytosols, while the estrogenic response was analysed either *in vivo* or in cell culture. Using intact cells has the advantage that the estrogen receptor is in its physiological environment with regards to the local receptor concentration and association with other cellular proteins, ionic strength, pH-value and temperature. Therefore, studies in a cellular system have the potential of being able to follow the fate and function of the estrogen receptor after ligand binding. For this reason, the breast cancer cell

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line MCF-7 has been serving as a model system [9–11]. These cells grow under hormonal control, have abundant estrogen receptors and provide parameters for functional assays, such as the induction of the progesterone receptor [9, 11, 12]. In these cells the addition of E_2 for periods of several hours leads to a loss of E_2-binding capacity, which was attributed to the activated receptor and termed processing [11–14]. Processing has also been associated with receptor protein degradation [15, 16] and correlated with the capacity of a ligand to induce estrogenic activity such as the induction of progesterone receptor synthesis [11, 14, 15].

While there is mounting knowledge on the biochemistry and molecular biology of the estrogen receptor, an aspect which has received less attention is the temporal requirement of the processes involved in the early signalling pathway to the estrogenic response. While changes in the binding parameters and the intracellular localization of the estrogen receptor have been described in the framework of minutes and hours, estrogenic responses were tested in the range of days. It has been shown that there is a rise in nuclear estrogen receptor 2–5 min after E_2 administration to the pig uterus; this was concomitant with a stochiometric increase in E_2 [8]. Even though the E_2 levels in the tissue decrease rapidly and the receptor protein is depleted during the first 5 h, long term effects such as an increase in the mitotic index were not observed until about 16 h later [2]. Similarly, in MCF-7 cells E_2 is bound to the cytoplasmic (extractable) receptor within 5 min and is subsequently found in the nucleus [11]. After 30 min there is a continuous loss of E_2-binding (processing) in both the cytoplasmic and nuclear fractions. The induction of the progesterone receptor, determined after 4 days of continuous E_2 exposure, showed a similar concentration-dependency as the loss of E_2-binding capacity [11]. These results are examples for the general observation that estrogenic responses are sustained after the steroid is no longer detectable.

Since changes in the estrogen receptor are detectable within minutes, the question arises how long the ligand is required to induce gene expression, detectable in a time frame of many hours or days. Therefore, in this study the time of commitment by E_2 to induce estrogenic effects in MCF-7 cells, namely the induction of the progesterone receptor and the stimulation of cell proliferation, was determined. To quantify the number of E_2 molecules required, the uptake of [^{3}H]E_2 was measured and the fraction of binding sites which could be occupied by a limited E_2 exposure was calculated. The results show that a 1 min E_2-pulse is sufficient to commit long term estrogenic effects and that only about 5% of the ER need to be occupied. During this short time receptor transformation as characterized by the loss of exchangeable E_2-binding sites was already induced.

MATERIALS AND METHODS

Cell culture

The human breast cancer cell line MCF-7 was received from G. Leclercq in 1988 and has been maintained in Richter's IMEM without phenol red (Biochrom), supplemented with 10% FCS (Serva) heat-inactivated for 1 h at 56°C. Trypsinized cells were plated at a titer of 4×10^4/ml and passaged weekly.

Induction of the progesterone receptor

Cells were transferred to medium containing 10% steroid-deprived FCS (i.e. FCS after treatment with charcoal-dextran at 0°C overnight, followed by a 2 h incubation at room temperature) for at least 3 days before adding E_2 at the indicated concentrations and for the indicated exposure times. E_2 was removed by changing the medium (with a quick intermittent wash with PBS, pH 6.5, at room temperature) to conditioned medium from untreated parallel cultures and incubating up to day 2. Progestin binding was then determined by incubating the cells with the synthetic progestin [^{3}H]ORG.2058 (1 nM) (Amersham) without and with 1 μM progesterone for 45 min at 37°C. Cell bound radioactivity was extracted with ethanol and counted in scintillation liquid [9, 12].

Determination of growth stimulation

Cells were maintained in medium with 10% CD-FCS 1 week before plating them in 35 mm dishes. E_2 was added to cell cultures in the same medium 24 h after plating for a brief exposure time (followed by a subsequent medium change as above) or for the whole duration of the 5 day incubation. The cell number was determined by allowing the cells to swell in hypotonic buffer ((20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$) and preparing a nuclei suspension by cell lysis with benzalkonium chloride (Janssen). The diluted suspension was counted in a Coulter Counter [9, 17]. Cell numbers of replicate cultures varied in the range of 10%.

Determination of estradiol-binding in whole cells

To determine the E_2-binding parameters by Scatchard analysis, [^{3}H]E_2 (Amersham) at concentrations ranging from 0.2 to 10 nM was added to semiconfluent cells in serum-free medium. Cultures were incubated for 30 min at 37°C. Cell-bound radioactivity was extracted from the cells with ethanol and counted in scintillation liquid, as described before [9, 12]. The scintillation count obtained with cells incubated with unlabelled 1 μM E_2 was subtracted as unspecific binding. For the kinetic studies, E_2 or [^{3}H]E_2 (depending on the protocol) was added to semiconfluent cultures plated in 10% steroid-deprived FCS and incubated at 37°C for the times indicated in the experiment. After changing the medium without or with addition of [^{3}H]E_2 or E_2, the cells were incubated

for another time interval at 37 C. Details are given in the figure legends. The bound radioactivity was extracted with ethanol as above.

Total cellular uptake of [^{3}H]E_2

[^{3}H]E_2 was added to subconfluent cells grown in medium with 10% steroid-deprived FCS and incubated for the indicated times. Cell lysates were prepared by adding hypotonic buffer (20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM dithiothreitol) to the monolayer on ice for 10 min; they were collected with a rubber policeman. Alternatively, cell monolayers were extracted with ethanol. With both procedures, radioactivity was determined by scintillation counting, and the results were similar.

Determination of estrogen receptors with monoclonal antibodies

Two monoclonal antibodies, D547 and H222, directed against the human ER [18], were used in a solid phase enzyme-immunoassay kit (ER-EIA, Abbott). Cell lysates were prepared as described for the uptake experiments, except that the buffer also contained 5 mM MoO_4 and 1 mM monothioglycerol instead of dithiothreitol. Aliquots were incubated according to the instructions of the assay kit. Briefly, an overnight incubation with antibody-coated beads was followed by an incubation with a second anti-ER antibody conjugated with horseradish peroxidase. The amount of estrogen receptor protein bound was determined by measuring the optical absorbance of the solution after reaction of *o*-phenylenediamine with the antibody-conjugated enzyme. The standard curve was determined in the same buffer as test samples.

RESULTS

Time of commitment for induction of estrogenic effects

E_2 at physiological concentrations (0.01–1 nM) induces progestrone receptor synthesis and stimulates proliferation of MCF-7 cells growing in steroid-deprived serum (CD-FCS) [9]. In these studies E_2 remained in the culture medium for the duration of experiment. Since incubations with E_2 for longer than 45 min induce receptor processing, i.e loss of binding capacity and receptor degradation [15], the question was what is the minimum exposure time required of E_2 to exert estrogenic effects.

In the following experiments the induction of progesterone receptor was determined after a 2 day incubation. This early time point was chosen to avoid loss of progesterone receptors as a result of the E_2 removal, since the level of the progesterone receptor is dependent on the presence of E_2 [11]. It was confirmed in a preliminary experiment that removal of E_2 did not result in loss of progestin binding within the 2 day incubation (data not shown). On the other hand, it was shown previously [9] that progestin binding begins to

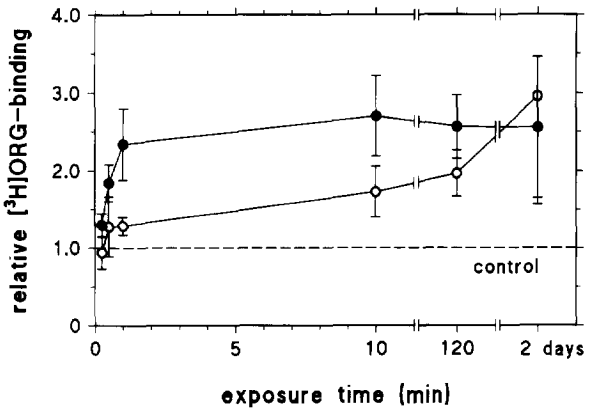

Fig. 1. Time of commitment by E_2 for the induction of the progesterone receptor. MCF-7 cells were exposed to (○) 0.1 nM and (●) 1 nM E_2 for the times indicated and medium was changed as described in Material and Methods. Cultures were incubated up to 2 days and assayed for progestin binding. Results are averages and SD (n) of 6–7 experiments. Control level of progestin binding in untreated MCF-7 cells was 4.8 fmol/10^5 cells.

rapidly increase about 8 h after E_2 addition. When MCF-7 cells received a single pulse of 1 nM E_2 for a time as short as 15 s, progestin binding was increased after 2 days, and with an E_2 pulse of 1 min the same stimulation was achieved as with a continuous incubation (Fig. 1). There was also a detectable stimulation of progestin binding after a 1 min pulse with E_2 at a concentration as low as 0.1 nM, but then more than 2 h were required to attain the same level of stimulation as with the continuous exposure.

The stimulation of MCF-7 cell proliferation was determined after a 5 day incubation. It also required a single pulse of 1 min or less with either 0.1 or 1 nM E_2 (Fig. 2). However, in contrast to the stimulation of progestin binding, exposures of more than 2 h

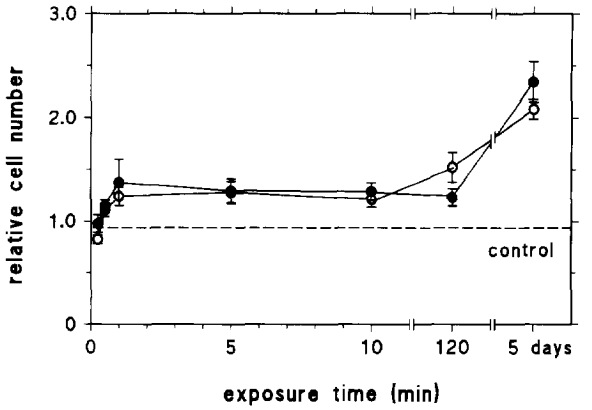

Fig. 2. Time of commitment by E_2 for the stimulation of cell proliferation. MCF-7 cells were exposed to (○) 0.1 nM and (●) 1 nM E_2 for the times indicated and removed as described in Material and Methods. Cell number was determined after a 5 day incubation. Results are the averages and SD (n) of 6–7 experiments. Control cell number was 5.0×10^5/35 mm culture dish.

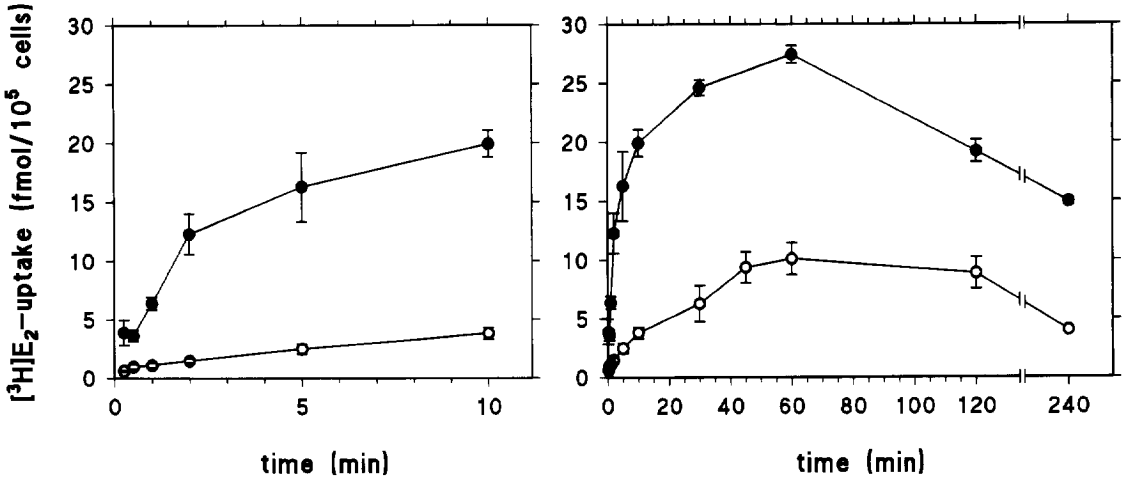

Fig. 3. Kinetics for uptake of E_2 at (○) 0.1 nM and (●) 1 nM by MCF-7 cell cultures. Data shows total binding. The same results are depicted for up to 10 min (left panel) and for up to 240 min (right panel). Averages and SD (n) are from 3 experiments.

were required to establish the maximal effect at both concentrations. Nevertheless, both the induction of the progesterone receptor and the stimulation of cell proliferation have in common that an exposure of the cells to E_2 for as short a 1 min is sufficient to commit the cells to induce the estrogenic response.

Cellular uptake of [^{3}H]E_2 by MCF-7 cells

How many E_2 molecules are taken up by the cell during this brief exposure time? The total number of cell-associated E_2 was calculated after various times of incubating MCF-7 cells with [^{3}H]E_2 at 0.1 and 1 nM (Fig. 3). There is a concentration dependent increase in bound [^{3}H]E_2 up to 60 min and a gradual decline thereafter, the latter being similar for both concentrations. After a 1 min incubation with 0.1 nM [^{3}H]E_2, about 7000 molecules of E_2 are bound per cell. With about 150,000 specific E_2-binding sites per MCF-7 cell determined by Scatchard analysis (Table 1), this number of E_2 molecules could occupy about 5% of the receptors. This fraction is apparently sufficient to stimulate more than a 5% increment of the estrogenic response (Figs 1 and 2). With 1 nM [^{3}H]E_2, about 38,000 molecules per cell were bound after a 1 min incubation, which is enough to occupy up to 25% of the receptors and to fully stimulate estrogenic responses.

Table 1. Changes in the K_d-value and the calculated number of E_2-binding sites (B_{max}) in MCF-7 cells after preincubation with 1 nM estradiol

Preincubation	K_d-value ($\times 10^{-10}$ M)	B_{max} ($\times 10^5$/cell)
None	4.10 ± 1.29	1.51 ± 0.50
E_2		
5 min	5.79 ± 2.34*	1.08 ± 0.49†
20 min	9.81 ± 4.31*	0.82 ± 0.34†
30 min	9.98 ± 1.94*	0.76 ± 0.22*
Overnight	13.97 ± 4.11‡	0.71 ± 0.44†

The results are averages and standard deviations [SD (n)] of determinations from 3–5 experiments. Significance of the K_d- and B_{max}-values in comparison with the control (no preincubation) was estimated with the paired t-test. †$P \leq 0.01$, *$P \leq 0.05$, ‡$P > 0.1$ (not significant).

Estrogen receptor dynamics

Are the short commitment times accompanied by changes in the E_2-binding characteristics of the MCF-7 cells? One possibility to examine changes in E_2 binding is to test for the exchangeability of bound E_2 after different times of preincubation. As Fig. 4 shows, increasing the time of the E_2 pulse leads to a continuous loss in the subsequent binding of [^{3}H]E_2, which is incubated for a constant 30 min. A pulse of 1 nM unlabelled E_2 for 1 min was sufficient to reduce the exchange with [^{3}H]E_2 by about 20%. The kinetics for

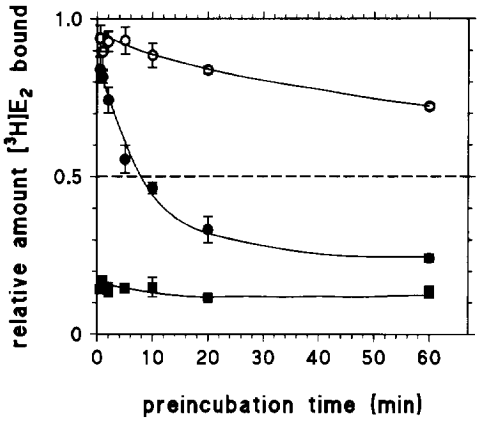

Fig. 4. Preincubation kinetics with E_2 leading to the loss of [^{3}H]E_2-binding. MCF-7 cells were incubated with (○) 0.1 nM, (●) 1 nM or (■) 1 μM E_2 for the times indicated. After removing the medium and washing, the cells were incubated with [^{3}H]E_2 at 0.2 nM added in serum-free medium for 30 min. Results are averages and SD (n) from 3 experiments.

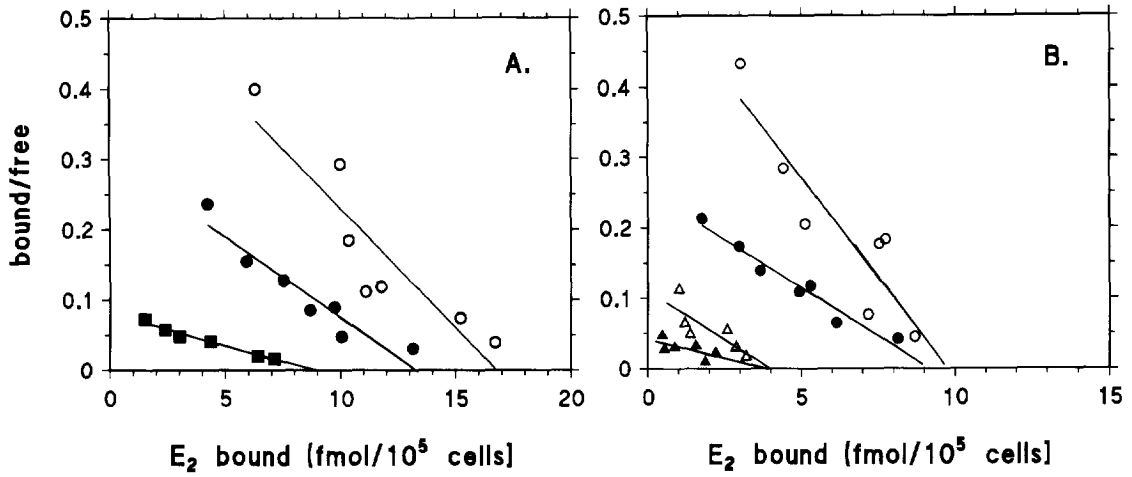

Fig. 5. Scatchard analysis of E_2-binding after preincubation with 1 nM E_2 (A). Cells were preincubated for (■) 30 min, or for (●) 5 min, or (○) left untreated before changing the medium and incubating with [^{3}H]E_2 for 30 min as described in Material and Methods (B). The effect of incubating with [^{3}H]E_2 for 10 min (●, ▲) or 30 min (○, △) on the binding analysis was compared for control cells (○, ●) and cell preincubated with 1 nM E_2 for 20 min (△, ▲).

the loss of exchangeable E_2 binding is concentration dependent; preincubation with 0.1 nM leads to a much slower reduction in the binding of [^{3}H]E_2. The reduction reached a plateau which was also dependent on the concentration of preincubated E_2.

Loss of exchangeable E_2-binding could be due to intracellular competition of [^{3}H]E_2 with the remaining unlabelled E_2 in the cell, and/or it could be due to a loss of binding sites. To distinguish between these possibilities, the binding data after different preincubation times was analysed by Scatchard plot. The shortest time of preincubation with E_2 was 5 min. It was followed by a 10 or 30 min incubation with [^{3}H]E_2 [Fig. 5(A and B)]. After the 5 min pulse, there was already a 30% loss of exchangeable E_2-binding sites. A longer preincubation time of 30 min further reduced the number of detectable binding sites. As shown by the subsequent incubation for 10 and 30 min, the determination of binding sites was not dependent on the time of incubation with [^{3}H]E_2. There was also an increase in the dissociation constant (K_d) with the preincubation time (Fig. 6, Table 1). The observed reduction in [^{3}H]E_2-binding after the E_2 pulse is, therefore, the result of both a loss of E_2-binding sites and a rise in the apparent K_d-value. From this data the reduced [^{3}H]E_2-binding after a 1 min pulse with 1 nM E_2 can be extrapolated to be due to an approx. 10% loss of the total number of exchangeable binding sites in the cell.

In most publications the loss of E_2-binding has been termed "processing" and has often been associated with loss or degradation of the receptor protein [15, 19, 20]. However, this phenomenon was observed with an E_2-incubation of at least 30 min or after several hours. The loss of binding sites as determined by Scatchard analysis of the binding data does not prove a loss of receptor protein. For this reason, the estrogen receptor protein was quantified by monoclonal antibodies against the human estrogen receptor [18] after various times of preincubation with E_2. Total estrogen receptor content was analysed in unfractionated cell lysates. Under these experimental conditions, the number of receptors detectable with the immunoassay correlated well with the number of E_2 binding sites determined by the Scatchard plot (Table 2). Preincubating MCF-7 cells with 1 nM E_2 for up to 30 min prior to the preparation of the lysate did not result in any loss of receptor protein detectable with these antibodies (Fig. 7). The slight increase in receptor protein at 30 min can be attributed to the greater stability of occupied compared to unoccupied estrogen receptors during the experimental manipulations. With longer E_2 preincubations, however, the number of receptor proteins was markedly reduced, as would

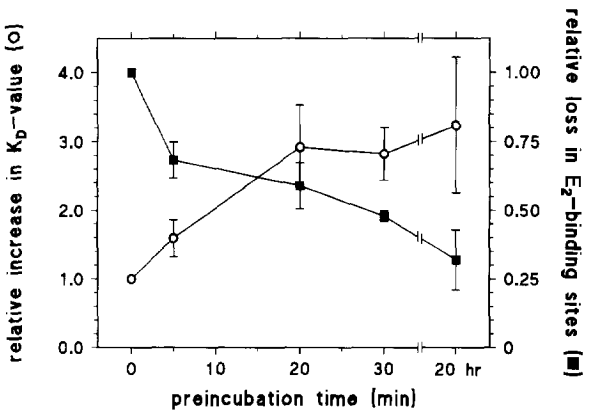

Fig. 6. Changes in the relative K_d values (○) and number of specific E_2-binding sites (■) with the time of E_2 preincubation. Results are the averages and SD (n) of 5 experiments. Absolute values are given in Table 1.

Table 2. Comparison of the number of E_2-binding sites with the number of estrogen receptor proteins in MCF-7 cells

Parameter	Method	Number/cell ($\times 10^5$)	n
B_{max}	Scatchard-Plot	1.32 ± 0.21	5
ER-protein	ER-EIA	1.28 ± 0.21	3

B_{max}, calculated maximal number of specific E_2-binding sites.
ER-EIA, estrogen-receptor enzyme-linked immunoadsorbent assay (Abbott) with whole cell lysates.
Averages and standard deviations [SD (n)] are shown.
MCF-7 cells were grown for 1 week in medium containing 10% steroid-deprived FCS before the beginning of the experiment.

be expected with the onset of receptor degradation. Taken together, these results demonstrate that in intact MCF-7 cells the initial loss of exchangeable binding sites following a brief E_2 exposure is not due to degradation of receptor protein, but more likely due to a conformational change of a fraction of the estrogen receptors to a high affinity binding state.

Inherent to the high affinity status of a fraction of the estrogen receptors is a slow rate of ligand dissociation. This should be measurable with MCF-7 cells after these had been preincubated with [^{3}H]E_2 for 5 or 30 min. Following the removal of the radiolabelled E_2, cells continued to be incubated without E_2. As shown in Fig. 8, the dissociation kinetics appear to have two phases. During the first, fast phase, the rate of dissociation following a 5 min preincubation is rapid (0.055/min) and about 10-fold faster than after a 30 min preincubation with [^{3}H]E_2 (0.0042/min). The initial loss of 3]E_2 can be attributed to the fraction of receptors remaining with exchangeable binding sites. In the

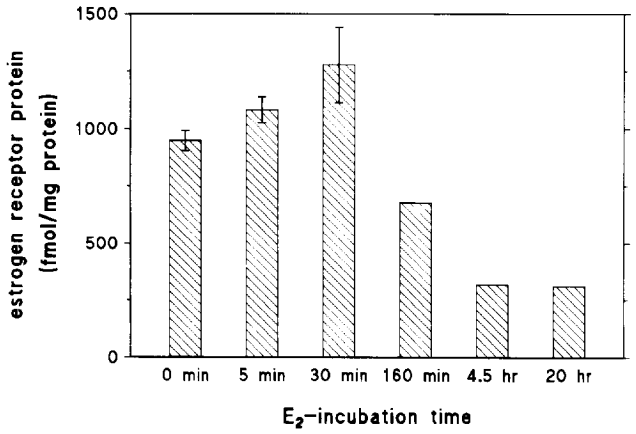

Fig. 7. Immunocytochemical determination of estrogen receptor protein in whole cells lysates of MCF-7 cells after incubating cells with 1 nM E_2 for the times indicated. Results for cells preincubated for 0, 5, and 30 min are averages and SD (n) from 3 experiments each with duplicate determinations. The results from the later time points are averages from a duplicate determination each from a single experiment.

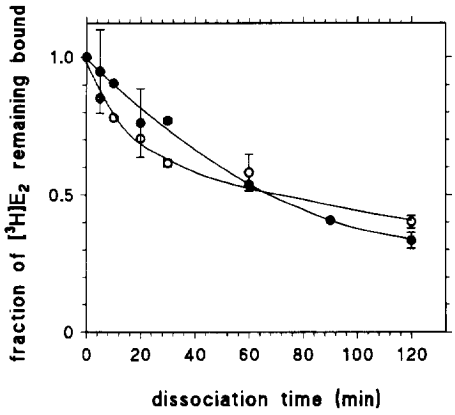

Fig. 8. Dissociation kinetics of [^{3}H]E_2. MCF-7 cells were preincubated with 1 nM [^{3}H]E_2 for (○) 5 min or for (●) 30 min. Following medium change, the cells were incubated without E_2 for the indicated times. Results are averages and SD (n) from 3 experiments.

second phase after about 60 min, the dissociation rates for [^{3}H]E_2 are very similar for both the 5 and 30 min preincubations (0.0023/min and 0.0035/min, respectively); these rates reflect the slow release of [^{3}H]E_2 from the high affinity receptor. The half life of this phase is in the range of 90–130 min. Similar values have been reported for the activated receptor in intact MCF-7 cells after a 50 min incubation with E_2 [12] and in uterine cytosols [21].

DISCUSSION

While numerous efforts have focussed on studying the biochemical changes and molecular biology of the estrogen receptor, the quantitative and temporal aspects of the receptor processes have been receiving less attention, It was, therefore, the goal of this study to correlate temporal changes in receptor binding with the induction of estrogenic effects, such as progesterone receptor synthesis and stimulation of cell proliferation. Here I show that (1) a 1 min exposure to 1 nM estradiol is sufficient to commit the MCF-7 cells to stimulate DNA-dependent estrogenic responses; and (2) this is accompanied by a loss of E_2-binding sites, which is due to the transformation of the estrogen receptor to a non-exchangeable, high affinity binding state.

It was calculated from the uptake experiment that at 0.1 nM E_2 about 7000 molecules are captured by the MCF-7 cells within 1 min, enough to occupy about 5% of the receptors. Since an increase in progestin binding was not observed before 12 h after E_2 addition [9] this suggests that the molecular species mediating the signal for transcription and translation is very stable. The transformation (activation) of the estrogen receptor has been studied extensively in various experimental systems. Receptor transformation is characterized by a change to a high affinity form with a slow dissociation rate of the bound E_2, by receptor

dimerization, increased nuclear binding, and it has been postulated to precede the activation of estrogenic response [1, 14, 21]. This view, was derived from experiments after incubation times of at least 30 min, more often after several hours. However, receptor transformation already occurs during the first minute of exposure to E_2 in the intact cell.

Processing of the estrogen receptor has been discussed as correlating with the manifestation of DNA-dependent responses by several authors [15, 20]. Depending on the experimental system, the term processing has been used with varying meanings. In general, it has described the loss of E_2 binding capacity after incubation times of several hours. Some studies, moreover, correlated processing with receptor transformation and degradation of the nuclear receptor [13–15].

In this study the initial loss of E_2-binding sites upon preincubation with E_2 was *not* due to receptor degradation. The loss of E_2-binding capacity after a 1 min ligand incubation complies with the definition of processing only if it is confined to changes in the binding parameters, since receptor degradation is not initiated before 30 min of incubation. It is therefore suggested that the term processing be confined to denote simply the loss of E_2-binding sites. Whether this is due to receptor transformation and/or receptor degradation will in each instance require further analysis. In any case, receptor degradation is a separate event and not *a priori* required to account for reduction of binding sites.

The experiments were explicitly done on intact cells to avoid loss of a possibly relevant subpopulation of estrogen receptor or its artificial redistribution. The absolute amounts of estrogen receptor detectable in cell homogenates depends on the extraction method [8]. Therefore, the immunoassay to detect the estrogen receptor protein was also done on whole cell lysates. Moreover, it has been reported by others that nuclear extraction and ultracentrifugation of the extracts is not necessary for quantitative detection of the receptor [22]. Indeed, using unfractionated samples for the binding and the immunological assays, I found a remarkable similarity in the number of specific E_2-binding sites and estrogen receptor-protein. This is in contrast to other studies using cytosols, which claim that the binding assay generally gives much lower values than the immunological determination of the estrogen receptor [23, 24].

The results presented here can be accommodated in the original "two-step" model for the action of the estrogen receptor at the cellular level, i.e. receptor transformation and translocation to the nucleus [1]. However, due to the risk of redistribution during cell fractionation, no attempt was made in this study to localize the activated estrogen receptor in the cell. The very short commitment-time for E_2 suggests that cytoplasmic or even membrane-associated estrogen receptors could be involved, which then translocate the E_2 to the nucleus. There is mounting evidence for a localization of the estrogen receptor in the microsomal fraction [25–27] and the plasma membrane [28–30]. A transport mechanism is supported by the observation that after a 2 min incubation of pig uteri *in vivo* with E_2, there is an increase in the immunogold-labelled estrogen receptor in the nucleus, with some label tagged to the nuclear pore complex [31]. The rise in the nuclear level of receptor was accompanied by a stochiometric rise in E_2 molecules, further suggesting that E_2 enters the nucleus with the receptor, or vice versa [2, 8].

The two parameters used to test the estrogenic response in MCF-7 cells, i.e. the induction of the progesterone receptor and the stimulation of cell proliferation, are obviously under different types of fine control, as apparent from the differences in the concentration dependence and the time required for the commitment of the maximal effect. This concept is supported by the fact that cell proliferation and induction of PgR are independently modulated by antiestrogens [11, 32]. While these two parameters have in common that a 1 min pulse of E_2 was sufficient for commitment, this brief exposure was capable of maximally stimulating progestin binding, but it was not sufficient for the maximal stimulation of cell proliferation. It has been reported that for maximal effects this exposure needed to be repeated during the course of cell growth [33]. It is remarkable that in spite of these regulatory differences, the initial signalling mode appears to be the same as evidenced by the time of commitment.

The brief exposure of cultured cells to E_2 resembles the *in vivo* situation in which free E_2 is rapidly removed from the blood by binding to proteins, especially sex hormone-binding globulins. In the organism, target tissue is not exposed to effective concentrations of estrogens continuously, but rather for specifically regulated times. Moreover, the short commitment time for inducing long term estrogenic effects and the rapid loss of E_2-binding sites appears to be a general property of estrogenic compounds. These characteristics have also been observed with synthetic nonsteroidal estrogenic compounds, e.g. with one derived from the synthetic hexestrol and coordinated to a platinum(II)-complex ([9], and unpublished data). On the other hand, the antiestrogen tamoxifen has neither the short commitment time for manifesting the growth inhibitory effect, nor does it lead to a loss of E_2-binding sites (manuscript in preparation). Thus the experimental protocol employed in this study is a step closer to the *in vivo* situation, and it also appears to be suitable to easily discern estrogenic from inactive and antiestrogenic compounds. However, many questions remain open concerning the temporal sequence and regulation of the processes involved in translating ligand binding into a DNA-mediated estrogenic response.

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REFERENCES

1. Jensen E. V. and DeSombre E. R.: Estrogen–receptor interaction. *Science* **182** (1973) 126–134.
2. Jungblut P. W., Hughes A., Gaues J., Kallweit E., Maschler I., Parl F., Sierralta W., Szendro P. I. and Wagner R.: Mechanisms involved in the regulation of steroid receptor levels. *J. Steroid Biochem.* **11** (1979) 273–278.
3. Gorski J., Welshons W. V., Sakai D., Hansen J., Walent J., Kassis J., Shull J., Stack G. and Campen C.: Evolution of a model of estrogen action. *Recent Prog. Horm. Res.* **42** (1986) 297–329.
4. Godowski P. J. and Picard D.: How to be both a receptor and a transcription factor. *Biochem. Pharmac.* **38** (1989) 3135–3145.
5. Beato M.: Gene regulation by steroid hormones. *Cell* **56** (1989) 335–344.
6. King W. J. and Greene G. L.: Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* **307** (1984) 745–747.
7. Welshons W. V., Lieberman M. E. and Gorski J.: Nuclear localization of unoccupied oestrogen receptors. *Nature* **307** (1984) 747–749.
8. Sierralta W., Bortsch A. C., Gaues J., Szendro P. I. and Jungblut P. W.: Estradiol-promoted accumulation of receptor in nuclei of porcine endometrium cells. Comparison of the retention of receptor in nuclei during subcellular fractionation of untreated and hormone-treated cells. *Receptor* **2** (1992) 17–28.
9. Otto A. M., Faderl M. and Schönenberger H.: Dissociation of estrogenic and cytotoxic properties of an estrogen receptor binding platin complex in human breast cancer cell lines. *Cancer Res.* **51** (1991) 3217–3223.
10. Lippman M., Bolan G., Monaco M., Pinkus L. and Engel L.: Model systems for the study of estrogen action in tissue culture. *J. Steroid Biochem.* **7** (1976) 1045–1051.
11. Horwitz K. B. and McGuire W. L.: Estrogen control of progesterone receptor in human breast cancer. *J. Biol. Chem.* **253** (1978) 2223–2228.
12. Olea-Serrano N., Devleeschouwer N., Leclercq G. and Heuson J-C.: Assay for estrogen and progesterone receptors of breast cancer cell lines in monolayer culture. *Eur. J. Cancer Clin. Oncol.* **21** (1985) 965–973.
13. Strobl J. S., Kasid A., Huff K. and Lippman M. E.: Kinetic alterations in estrogen receptors associated with estrogen receptor processing in human breast cancer cells. *Endocrinology* **115** (1984) 1116–1124.
14. Kasid A., Strobl J. S., Huff K., Greene G. L. and Lippman M. E.: A novel nuclear form of estradiol receptor in MCF-7 human breast cancer cells. *Science* **225** (1984) 1162–1165.
15. Gyling M. and Leclercq G.: Estrogen and antiestrogen interaction with estrogen receptor of MCF-7 cells—relationship between processing and estrogenicity. *J. Steroid Biochem.* **29** (1988) 1–8.
16. Borras M., Hardy L., Lempereur F., El Khissiin A. H., Legros N., Gol-Winkler R. and Leclercq G.: Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression. *J. Steroid Biochem. Molec. Biol.* **48** (1994) 325–336.
17. Butler W. B.: Preparing nuclei from cells in monolayer cultures suitable for counting and for following synchronized cells through the cell cycle. *Anal. Biochem.* **141** (1984) 70–73.
18. Greene G. L., Sobel N. B., King W. J. and Jensen E. V.: Immunochemical studies of estrogen receptors. *J. Steroid Biochem. Molec. Biol.* **20** (1984) 51–56.
19. Horwitz K. B. and McGuire W. L.: Nuclear estrogen receptors. Effect of inhibitors on processing and steady state levels. *J. Biol. Chem.* **255** (1980) 9699–9705.
20. Gyling M. and Leclercq G.: Estrogenic and antiestrogenic down-regulation of estrogen receptor levels: evidence for two different mechanisms. *J. Receptor Res.* **10** (1990) 217–234.
21. Weichman B. and Notides A. C.: Estradiol-binding kinetics of the activated and nonactivated estrogen receptor. *J. Biol. Chem.* **252** (1977) 8856–8862.
22. Hähnel R., Avenell M. H. J. and Hähnel E.: Estrogen and progesterone receptor assays, by the EIA method (Abbott), of low-speed supernates of breast-cancer homogenates. *Clin. Chem.* **35** (1989) 2226–2227.
23. Kral L. G., Doherty L. M. and Brooks S. C.: Quantitative determination of nuclear estrogen receptors by an enzyme immunoassay: applicability and caveats. *J. Steroid Biochem.* **31** (1988) 459–466.
24. Marsigliante S., Puddefoot J. R., Barker S., Gledhill J. and Vinson G. P.: Discrepancies between antibody (EIA) and saturation analysis of oestrogen receptor content in breast tumour samples. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 643–648.
25. Little M., Szendro P., Teran C., Hughes A. and Jungblut P. W.: Biosynthesis and transformation of microsomal and cytosol estradiol receptors. *J. Steroid Biochem.* **6** (1975) 493–500.
26. Muldoon T. G., Watson G. H., Evans A. C. Jr and Steinsapir J.: Microsomal receptor for steroid hormones: functional implications for nuclear activity. *J. Steroid Biochem.* **30** (1988) 23–31.
27. Steinsapir J. and Muldoon T. G.: Role of microsomal receptors on steroid hormone action. *Steroids* **56** (1991) 66–71.
28. Nenci I., Marchetti E., Marzola A. and Fabris G.: Affinity cytochemistry visualizes specific estrogen binding sites on the plasma membrane of breast cancer cells. *J. Steroid Biochem.* **14** (1981) 1139–1146.
29. Berthois Y., Pourreau-Schneider N., Gandilhon P., Mittre H., Tubiana N. and Martin P. M.: Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. *J. Steroid Biochem.* **25** (1986) 963–972.
30. Germain P. S., Metezeau P., Tiefenauer L. X., Kiefer H., Ratinaud M. H. and Habrioux G.: Use of a biotinyl-estradiol derivative to demonstrate estradiol-membrane binding sites on adherent human breast cancer MCF-7 cells. *Anticancer Res.* **13** (1993) 2347–2353.
31. Sierralta W., Jakob F., Thole H., Engel P. and Jungblut P. W.: Estradiol-promoted accumulation of receptor in nuclei of porcine endometrium cells. Immunogold electron microscopy of resting and estradiol-stimulated cells. *Receptor* **2** (1992) 29–37.
32. Eckert R. L. and Katzenellenbogen B. S.: Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. *Cancer Res.* **42** (1982) 139–144.
33. Marth C. and Daxenbichler G.: Pulsatile versus continuous estradiol exposure in inducing proliferation of cultured ZR-75.1 human breast cancer cells. *J. Steroid Biochem.* **23** (1985) 567–572.