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Review

Calcium-induced activation of estrogen receptor alpha – New insight

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ABSTRACT

Calcium being an important modulator in multiple regulatory processes, we overviewed reported investigations concerning its potential influence on ERα transcriptional activity in breast cancer cells. Three main activating mechanisms depending on either intra- or extracellular calcium are described. At physiological intracellular concentration (μM), Ca^{++} activates calmodulin and promotes its association with ERα; the resulting complex stably interacts with EREs at promoter sites, giving rise to enhanced transcription of estrogen target genes. Hypercalcemic concentrations (mM) produce a similar response through a direct association of the ion with the ligand binding domain of the receptor, this binding of calcium conferring an active conformation to ER. In contrast to these intracellular processes, very high extracellular concentrations of Ca^{++} (>10 mM) detected in bones at time of tumor metastasis operate via a signal transduction pathway initiated at the cell membrane through a specific activation of the calcium-sensing receptor.

Contents

1. Introduction

Although Steroid Hormone Receptors belong to the nuclear receptor family, their cellular localization is not restricted to the nucleus where they operate as ligand-dependent transcription factors. Receptors apparently recruited at the cell membrane elicit quasi-immediate responses under hormonal stimulations. In fact, functions of nuclear and membrane receptor pools are less independent than originally thought: the regulation of one pool usually modulates the activity of the other. The finding that these pools, as well as a few associated coregulatory proteins, are subjected to a permanent subcellular trafficking under the action of various intra- and extracellular modulators, is most probably a major determinant of this interrelationship [1–5].

To decrypt the underlying molecular mechanisms of this complex regulatory process is a real challenge. Calcium being a well-known modulator of multiple intracellular mechanisms, we intended to evaluate its potential contribution. Since investigations of our team focused on wild type 67 KDa ERα in breast cancer cells, we restricted our analysis to this receptor.

Whether or not calcium may influence ERα function is not a new question. Already, in the late 60's/early 70's, several authors described a Ca^{++}-dependent proteolysis of the receptor, which abrogates its ability to dimerize and associate with regulatory partners [6–8]. The physiological pathophysiological relevance of this degradative activity which operates on the hinge domain of the receptor remained, however, controversial because it required a supraphysiological concentration of Ca^{++} (mM). Subsequent investigations in the 90's provided more convincing data in favor of a possible interference of the ion in ERα-dependent functions. Thus, treatment of MCF-7 breast cancer cells with the A23187 Ca^{++}

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ionophore was reported to downregulate ERα mRNA, inducing thereby a depletion of the receptor [9]. Moreover, incubation of these cells under a hypercalcemic condition (2.5 mM Ca^{++}) was found to decrease ERα level [10], leading to the concept that variations in intra- and extracellular calcium levels may regulate responses to estrogenic stimuli, at least in breast cancers. More recent studies confirm this statement and reveal three distinct mechanisms by which Ca^{++} may enhance ERα-mediated transcriptions. It is our purpose to overview these mechanisms.

2. Targets conferring an ability to calcium to enhance ERα-mediated transcription in breast cancer cells

As illustrated in the Fig. 1, it is now established that intracellular calcium at physiological concentrations (μM) confers to calmodulin (CaM) an appropriate conformation to interact with ERα in the cell nucleus; the resulting complex stably associates with Estrogen Response Elements (EREs) at promoter sites to enhance receptor-mediated transcription [11]. At supraphysiological concentrations (mM), Ca^{++} may interact with the ligand binding pocket of ERα to induce the activation of the latter and increase its transcriptional activity [12]. Finally, high extracellular Ca^{++} concentrations found in bone tissue microenvironment (>10 mM) at time of bone metastasis similarly enhance ERα-mediated transcription, through an interaction with a specific calcium sensing membrane receptor [13]. Hence, three distinct Ca^{++} targets may confer to this ion an ability to regulate ERα activity. Next sections describe mechanisms by which these targets operate.

2.1. Calmodulin

Assessing the role of ERα phosphorylation at Y_{537}, G. Castoria of F. Auricchio's group discovered an association between the receptor and CaM [14]. Hence, the discovery of this association must be ascribed to these investigators even if they did not elucidate its function. To my knowledge, my team was the first to report that CaM stabilizes the complexion of ERα with EREs, suggesting its contribution to receptor-mediated transcription [11]. This suggestion was confirmed by Biswas et al. [15], as well as by Ramos [16], before it was definitely established by Sacks as a result of extensive investigations [17].

Studies conducted in our laboratory [18] revealed that CaM strongly interact with four basic residues ($K_{299}RSKK_{303}$) of a regulatory platform of ERα located between P_{295} and T_{311}, this sequence

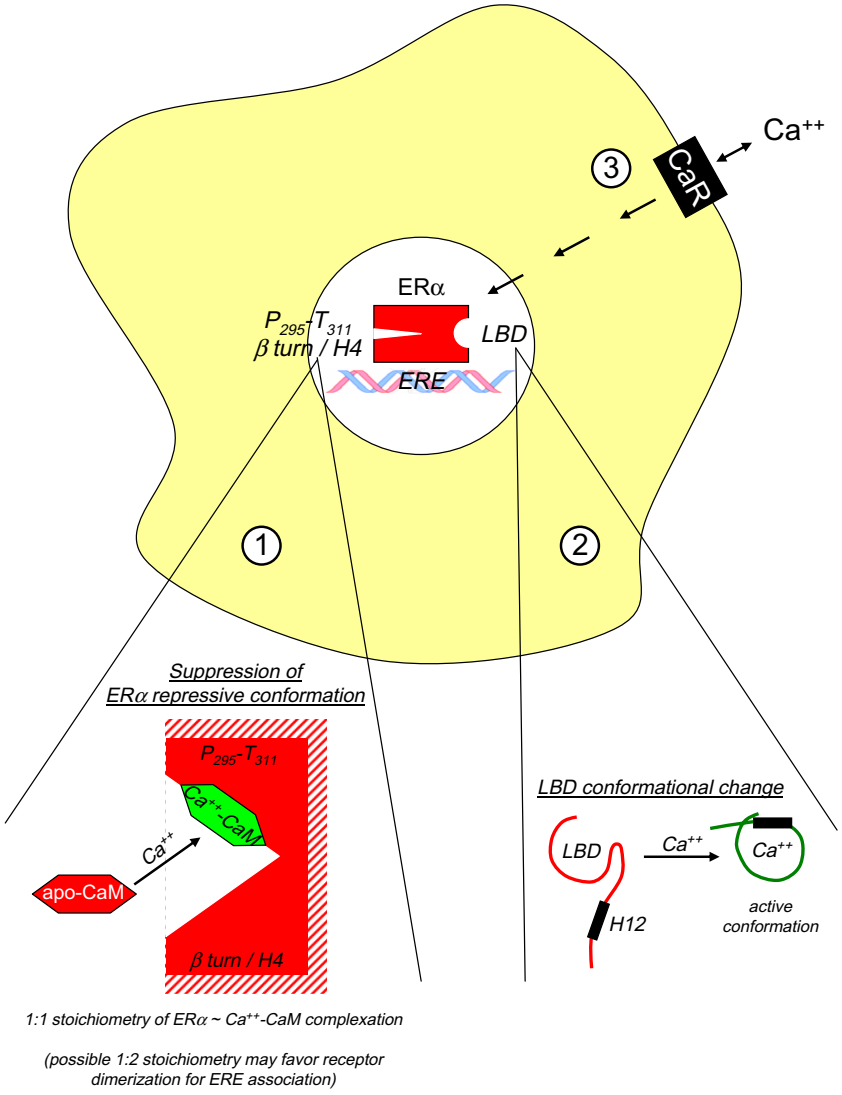

Fig. 1. Mechanisms by which physiological (1) and supraphysiological (2) intracellular Ca^{++} concentrations confer to ERα an appropriate conformation to enhance receptor-mediated transcription in MCF-7 cells. At very high extracellular concentrations, Ca^{++} operates through an interaction with its CaR membrane sensing receptor (3).

being just in front of the ligand binding domain (LBD). This P_{295}–T_{311} sequence interacts with a motif (H4/β turn) of the LBD, suggesting auto-regulatory properties; interaction with ERα regulatory partners may also be considered because it is subjected to various posttranslational modifications required for receptor trafficking, transcriptional activity and turnover. Our finding that the deletion of this sequence (mutagenesis) increases the basal transcriptional activity of the receptor revealed its implication in a repressive mechanism that would be abrogated, at least in part, by CaM binding.

Requirement of Ca^{++} for the formation of ERα–CaM complex, detected in the past by conventional biochemical approaches [11,14–18], has been recently confirmed by Electron Spray Ionization-Mass Spectrometry (ESI-MS) using a synthetic peptide (ERa17p) corresponding to the P_{295}–T_{311} sequence, instead of the whole receptor which is too large for such an analysis [19]. This study, conducted by J.C. Tabet (University P&M Curie, Paris) with whom we collaborate, indicated that about 75% of ERα17p fail to associate with CaM in absence of Ca^{++}; residual 25% of peptide associated according to a 1:1 stoichiometry. As expected, levels of Ca^{++} bound to CaM strongly influenced its ability to associate with the peptide: abundance of 1:1 ERα17p–CaM complex reached values of about 40% and 55% in the presence of two and four ions, respectively. Moreover, in the presence of four bound Ca^{++}, an additional complexation involving two peptides (15%) was recorded. ERα17p concentration also influenced complexation: under optimal Ca^{++} condition (4 bound Ca^{++}), the 1:1 stoichiometry occurred at a peptide concentration about five times lower than required for the 1:2 complexation. Such a 1:2 stoichiometry being quite unusual, a control experiment was carried out with melittin, a well known CaM-binding protein. Remarkably, melittin concentration also influenced stoichiometry (100% of melittin associated with the $4Ca^{++}$/CaM complex according to a 1:1 stoichiometry at low concentration while almost identical amounts of 1:1 and 1:2 complexes were recorded at higher concentrations), clearly indicating that the association mode of ERa17p with CaM is not unique. Although such a 1:2 stoichiometry is not established for ERα, one may speculate upon its potential existence and biological significance: it may reflex a capacity of CaM to favor receptor dimerization required for ERE binding and related transcription.

Present study suggests that physiological Ca^{++} oscillations governing CaM activity may act as a metronome, regulating basal ERα transcriptional activity and contributing to its well known cyclic character [20]. Note in this context that CaM has been shown to protect ERα against proteasomal degradation [21]. Hence, deactivation of CaM because of a cyclic decrease of Ca^{++} concentration would favor a destabilization of the ERα–CaM complex at promoter sites, provoking a release and degradation of the mature ERα which has already performed its function in the transcription cycle. One may suspect that such a step may favor the recruitment of a newly synthesized receptor pool able to sustain the initiation and progression of new transcription cycles required for a significant biological response [3,22].

2.2. ERα ligand binding domain

Very recently Martin and coworkers reported a binding of radioactive $^{45}Ca^{++}$ to purified recombinant preparations of ERα and corresponding LBD [12]. Binding affinity of this complexation was about 1000 lower than measured with E_2(Kd ∼ 1 μM). Site-directed mutagenesis and molecular docking studies based on the crystal structure of the LBD allowed the identification of aminoacid residues implicated in this interaction. Although these residues are not the same as those recognizing the hydroxyl functions of E_2 required for the onset of a biological response, they provoke a conformational change of the LBD similar to the one described for the hormone. Once Ca^{++} is inserted within the ligand binding pocket, H12 is displaced in such a way that it covers the engulfed ion and unmasks binding motifs for coactivators.

This Ca^{++}-induced conformational change may explain our previous finding that a pretreatment of a purified recombinant ERα (and corresponding LBD) with Ca^{++} at the millimolar concentration strongly decreases the capacity of the receptor to bind $[^3H]E_2$ as well as other ligands [23]. The observation that this loss did not occur with the ^3H-labeled receptor suggested to us a binding process in which the inserted ligand impedes its displacement by another ligand, which is in agreement with the displacement of H12 just described. Moreover, the weak binding affinity of Ca^{++} for ERα with regard to E_2 explains why very large excess of Ca^{++} are required to affect $[^3H]E_2$ binding.

Martin revealed that this Ca^{++}-induced conformation change of the LBD enhances ER-mediated transcription [12]. Incubation of COS-1 cells transiently transfected with ERα under hypercalcemic conditions (5 mM) increased its transcriptional activity. The observation of a similar phenomena with EGF (150 ng/ml) which increases the intracellular Ca^{++} concentration by the PLC-γ pathway, supports this view (abrogation of transcription increase by a PLC-γ inhibitor). The use of two truncated ERα constructs devoid respectively of AF1 (AB domain/HEG19) and AF2 (LBD/HE15) confirmed that such transcription enhancement may be ascribed at least in part to an effect of Ca^{++} on the LBD: both constructs gave similar responses. The fact that such observations were associated with an increase of intracellular Ca^{++} concentration supports the concept of a direct association of the ion with the receptor (HEG19), without excluding additional indirect contribution based on interferences in signal transduction pathways (HE15).

In this work, Ca^{++} and EGF were reported to similarly induce expression of PgR and pS2 in MCF-7 cells, clearly indicating that the ERα activating potency of Ca^{++} modulates the expression of endogenous genes [12]. In fact, in a previous study devoted to the analysis of the effect of high amounts of Ca^{++} on ERα regulatory properties in MCF-7 cells (see below), we also recorded a PgR induction under an almost equivalent hypercalcemic culture condition [13]. This property was tentatively ascribed to a unique indirect activation process initiated at the cell membrane.

2.3. Membrane Ca^{++} sensing receptor

Since metastatic breast tumor cells in bone may be exposed to very high amounts of Ca^{++} (≫10 mM), we evaluated the potential effect of increasing Ca^{++} concentrations on ERα-mediated transcription [13]. MCF-7 cells stably transfected with an ERE-dependent reporter gene (MVLN cells) were selected for that purpose [study conducted in cooperation with J.J. Body; bone disease Dept.]. We found that this increase induces a progressive enhancement of transcriptional activity as well as a strong depletion of the receptor, as found under stimulation with E_2. At the highest tested Ca^{++} concentration (20 mM), these effects were extremely well marked. A lack of similar response with cells exposed to the A23187 ionophore rejected the possibility of an influx of Ca^{++} being at the origin of our observations; rather, we speculated upon a potential implication of a signal transduction pathway initiated at the cell membrane through an activation of a specific Ca^{++} target. The use of two calcimimetics (NPS R-467 and NPS S-467) acting via the calcium-sensing receptor (CaR) confirmed our view. At 0.1 μM, both compounds suppressed the effect of 20 mM Ca^{++} on ERα transcriptional activity and depletion, with an efficiency closely related to their reported stereoselectivity. The fact that a specific antagonist of CaR inhibited at 1 μM all effects of 20 mM Ca^{++} left no doubt that the ion operates at high concentration on breast cancer cells, at least in part via its membrane receptor.

3. Conclusion

Present investigations reveal that calcium is an important modulator of ERα activity in breast cancer cells. While studies reported here mainly focus on receptor-mediated transcriptions, one may speculate that the ion may influence many other functions, especially those depending on membrane and cytoplasmic receptor pools, as described in other cell types. As this topic has not been extensively addressed so far, we encourage investigators to initiate pilot studies to evaluate the potential impact of these pools. The use of stable Ca^{++}-dendrimers (or any other soluble macromolecular conjugates) unable to cross the nuclear membrane is an especially attractive approach for such a task. While awaiting the synthesis of such compounds, we propose to test the effect of Ca^{++} solutions containing agents known to modify the impact of shuttling/trafficking properties of ERα on both cell growth and transcription of reporter genes (for example, 2-bromo-hexadecanoic acid which abrogates the association of the receptor with the plasma membrane [24], leptomycin B which maintains its localization within the nucleus [25]). In this context, the use of ERα mutated at sites implicated in intracellular movements of the wild type receptor may also be proposed. Among such mutations, substitution of Ala-447 for Cys-447, which abrogates ERα palmitoylation required for its association with the plasma membrane, appears of prime importance because this association is mainly responsible of receptor-initiated transduction pathways [24].

On the other hand, data reported in this review support the concept that a loss of Ca^{++} homeostasis may concur to breast cancer emergence and development [26]. Identification of genes clusters related to oncological processes by conventional microarray technology in breast biopsies from patients diagnosed with breast cancer would be a strong argument for this concept, stressing a need for such an analysis. Confirmation of such a suspected relationship would be a starting point for experimental studies aimed to decrypt the underlying mechanism. In this regard, one may speculate that the observation of an enhanced expression of these peculiar genes clusters in mammary cell lines cultured under conditions favoring one of three Ca^{++} activating mechanisms of ERα-mediated transcription described below, may be particularly informative. Data from these investigations would logically be integrated in a drug design program for the production a new class of ERα (or ERβ, if the latter proves to be subjected to Ca^{++} activation) antagonists.

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