

EFFECT OF Ca⁺⁺ AND Mg⁺⁺ ON THE BINDING OF CYTOPLASMIC AND NUCLEAR ESTRADIOL RECEPTORS IN BREAST CANCER OF FEMALE PATIENTS

By

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تأثير الكالسيوم والماغنسيوم على ترابط مستقبلات الاستروجين في السائل الخلوي والنواه للسيدات المرضى أحمد صبحي الشراكي و كمال محمد قنديل نبيل أحمد منصور و مها محمد صبري

تناول البحث تعيين مستقبلات هرمون الاستراديول في السائل الخلوي والنواه لأربعة عينات من أنسجة ثدي لسيدات مصابات بسرطان الثدي وعينة واحدة من رحم طبيعي . وقد وجد أن كمية هذه المستقبلات في السائل الخلوي يتراوح بين ١,٤ × ١٠^{-٣} و ٢,٦ × ١٠^{-٢} بيكومول/مليجرام بروتين ، بينما كانت كمياتها أكبر في النواه بالمقارنة بالسائل الخلوي (٠,٠٨٩ - ٢,٤٩) بيكومول/مليجرام بروتين . وقد وجد أن ثابت التفكك لمستقبلات الاستراديول أعلى في جزئيات النواه عنها في السيتوسول للأنسجة السرطانية . كما وجد أن إضافة الكاتيونات ثنائية التكافؤ (الكالسيوم والمغنسيوم) لجزئيات النواه يغير من خاصية الارتباط لهذه المستقبلات في عينة نسيج الرحم بينما يغير من خواصها الطبيعية المصاحبة في الأنسجة السرطانية وقد لوحظ أنه بإضافة الكاتيونات يحدث زيادة ملحوظة في قدرة مستقبلات النواه على الارتباط في الأنسجة السرطانية ، بينما لم يلاحظ مثل هذه الزيادة في السائل الخلوي للأنسجة السرطانية أو عينة الرحم . ومع ذلك فإن سلوك وقابلية هذه المستقبلات في الأورام المرتدة نحو فعل هذه الكاتيونات كان مختلفاً عنه في الأنسجة السرطانية الأخرى التي تم دراستها .

Key Words: Breast cancer, Estradiol receptors, Effect of Ca⁺⁺ and Mg⁺⁺

ABSTRACT

Specific estradiol receptors were quantitatively determined in the cytosol and nuclear fractions of four cancerous breast tissues and one normal uterus. Validated methods are described for estradiol receptor assay. The number of cytosol receptors was observed to be in the range of 4.1×10^{-3} - 6.2×10^{-2} pmoles/mg protein, while the number of nuclear receptors was significantly higher compared with cytosol receptors (0.089 to 2.49 pmoles/mg protein). The dissociation constant (K_d) of the estradiol receptors, analyzed from the Scatchard plot, was higher in the nuclear pellets than in cytosols of cancerous. The addition of divalent cations (Ca²⁺ or Mg²⁺) to the nuclear preparation, altered the binding properties of the receptors in the human uterus; while they altered their physical association state in the cancerous tissues. In the presence of cations, marked activation of the nuclear binding in the cancerous tissues was observed; a pattern which was not clearly observed in the cytosols of the same tissues or in the uterus. However, the receptor's affinity and behaviour of a recurrent tumor, towards the action of these cations were different from that of other investigated tissues.

INTRODUCTION

Kennedy (1965) and Segaloff *et al.*, (1972) reported that hormonal manipulation of the patients, in the form of either removal of the source or addition of steroid hormones, has been observed to cause remission of breast cancer. However, not all the breast tumors respond to hormonal manipulation.

Extensive investigations made by Jensen and DeSombre (1973) on the role of steroid hormone receptors in the response of the target tissues in rat and calf to 17β -estradiol led to the proposal that there is a possible correlation between the presence of estradiol receptor protein (ER) and regression in human breast carcinomas following endocrine manipulation (Jensen *et al.* 1971).

One of the major problems in the treatment of breast cancer, is the ability to differentiate between hormone dependent and independent tumors with the help of predictive tests. McGuire *et al.*, (1975) and Savlov *et al.*, (1974) stated that the assay of estradiol receptor protein is currently accepted as one of the basis for discrimination between these two kinds of tumors.

It has been observed by Hodge *et al.*, (1973) and McGuire *et al.*, (1977) that estradiol receptor positive tumors respond favourably to endocrine therapy and vice versa. It has, however, become evident, as found by McGuire *et al.*, (1975) and McGuire and Horwitz (1975), that not all the cytosol estradiol receptor positive tumor respond to endocrine therapy, and that there is no need to develop a more accurate test to predict the hormonal responsiveness of the breast tumor.

So far, the estradiol receptor assay has been limited to the estimation of estradiol receptors in the cytosol fraction, without taking into consideration the number of cytosol receptors that get transported to the nucleus to be of functional importance at the nuclear level.

On the receptor molecular bases and its relation to several cations, Schneider and Dao (1977) suggested that cytosol of human mammary tumor may contain proteolytic activities that can be activated by Ca^{2+} and that can effect irreversible changes in the salt dissociated steroid receptor proteins.

Thus, the present study was undertaken with two main objectives. First, comparison between the cytosol and nuclear estradiol receptors in the human uterus and cancerous breast tissues. Second, study the effect of divalent cations (Ca^{2+} and Mg^{2+}) on the estrogen receptor capacity to bind (^3H)-estradiol.

MATERIALS AND METHODS

Chemicals:

(2, 4, 6, 7, 16- ^3H)-Estradiol in toluene/ethanol (9:1) solution with specific activity 104 Ci/m mol and $^{45}\text{CaCl}_2$ solutions with specific activity 0.25 m Ci/ml obtained from Radiochemical Center, Amersham, England. 17β -Estradiol, was obtained from Sigma Chemical Co., USA.

Tris - EDTA dithiothreitol (TED) buffer (pH 7.4), 10 mM Tris-HCl buffer contained, 1.5 mM EDTA, and 0.5 mM dithiothreitol. Sucrose-Tris buffer I (pH 7.8), contained 1mM MgCl_2 , 3 mM CaCl_2 , 10 mM KCL, 1 mM dithiothreitol, 250 mM sucrose, in 20 mM Tris-HCl. Sucrose-Tris buffer II(pH 7.8), contained 10 mM KCl, 1 mM dithiothreitol, 250 mM sucrose, in 20 mM Tris-HCl buffer.

The dextran-coated charcoal suspension, (pH 8) contained 0.0025% dextran (Pharmacia) and 0.025% charcoal (Norit A, Sigma) in 10 mM Tris-HCl buffer.

Endometrial Tissues:

Endometrial samples were obtained by curettage from postmenopausal patients consulting for gynecological disorders with or without minor menstrual disturbances. Wet tissue was dipped into ice-cold isotonic saline to remove mucus, blood clots and sometimes myometrial pieces. It was then prepared for deep freezing.

Tumor Tissues:

Four pathologically confirmed, as infiltrating duct mammary carcinoma type, tissues were obtained from surgical specimens of breast cancer postmenopausal patients undergoing mastectomy and designated as case A., B., C and D. Tissue samples were obtained from each surgical specimen immediately after mastectomy and was prepared for deep freezing as above.

Preparation of Cytosol and Nuclear Fractions:

Tissues were prepared according to the procedure described by Wilson *et al.*, (1980). All subsequent operations were made at 0-4°C. The tissue was homogenized in 4 to 10 ml of TED buffer per gram tissue, by using a polytron. The homogenate was centrifuged at 800 xg for 10 minutes. The 800 xg supernatant was taken centrifuged at 105,000 xg for 60 min. to separate the "cytosol fraction". The 800 xg nuclear pellet was resuspended in 1 ml sucrose-Tris buffer I and gently rehomogenized by hand in a glass homogenizer. The nuclear suspension was recentrifuged at 800 x g for 10 minutes, washed twice and resuspended in the same buffer. Protein contents of both cytosol and nuclear pellet was quantitated by the method of Lowry *et al.*, (1951).

Estrogen-Receptor Assay

Triplicate aliquots of (100 μl) of cytosol or nuclear pellet together with 200 μl of homogenizing buffer were incubated with increasing concentrations of (^3H)-estradiol for 18 hrs at 4°C. Fifty microliters of 100-fold excess of 17β -estradiol was used to determine the non-specific binding. Solutions of divalent cations were added as 100 μl aliquots of (10^{-5} and 10^{-3} M) to these tubes in a second assay.

The free and loosely bound (^3H)-estradiol in the cytosol was then removed by dextran-coated charcoal (Wilson *et al.*, 1980).

In the case of nuclear pellet, the incubation mixture was filtered over Whatman GF/B glass fiber filter (pore size 1.0 μm) and immediately washed with 10 ml of cold sucrose-tris buffer II. The filter was used to separate the nuclear pellet's bound- from free-(^3H) estradiol activity. (El-Defrawi *et al.*, 1980; Alexander *et al.*, 1987). The filter was then taken for liquid scintillation counting.

Radioactivity Measurement

Cytosol samples and the filter of the nuclear pellets were counted in 10 ml custom cocktail (0.75 g PPO, 0.32 g dimethyl POPOP, and 40 ml of Beckman BBS solubilizer per liter toluene) using a liquid scintillation spectrometer. Counts

were corrected to 100% efficiency by external standardization. The data were analyzed by the method of Scatchard (1949).

Effect of Cation's Addition on (³H)-Estradiol Binding to Cytosol and Nuclear Estradiol Receptors:

Fifty microliters cytosol or nuclear pellet preparation were added to 100 μl of calcium or magnesium solutions with concentrations from 10⁻⁹ to 10⁻² M. One hundred microliters (³H)-estradiol (0.5 pmole/200 μl TED buffer) were added to all tubes. The volumes were completed to 1 ml with TED or sucrose-tris buffer II.

RESULTS AND DISCUSSION

In this work, estradiol binding was determined at six (³H)-estradiol concentrations and the results were plotted in Scatchard plots. Cytosol (or nuclear fraction) of case D (Fig. 1) as well as human uterus (Fig. 2) gave a linear relationship in which the intercept on the abscissa gives the number of binding sites (B_{max}), while 1/slope of the line gives the affinity of the receptor. The cytosol of case B (Fig. 3) gave a straight line parallel to the abscissa, i.e., the proportion of estradiol bound was constant and independent of the estradiol concentration in the incubation medium, and no saturation of binding sites could be demonstrated. In contrast, a non-linear curve which indicates the presence of more than one phase of binding mode was obtained in the cytosol of the two mostly high-binding carcinomas (cases A and C) (Figs. 4 and 5).

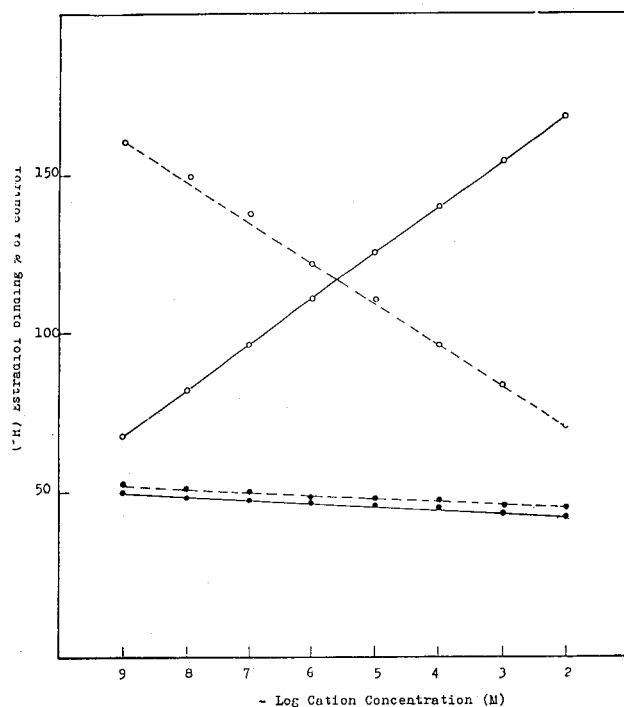


Fig. 2: Effect of cations on (³H)-estradiol binding to cytosol (•) and nuclear (o) receptors from the uterus as a reference tissue [(-) Ca²⁺ and (...) Mg²⁺].

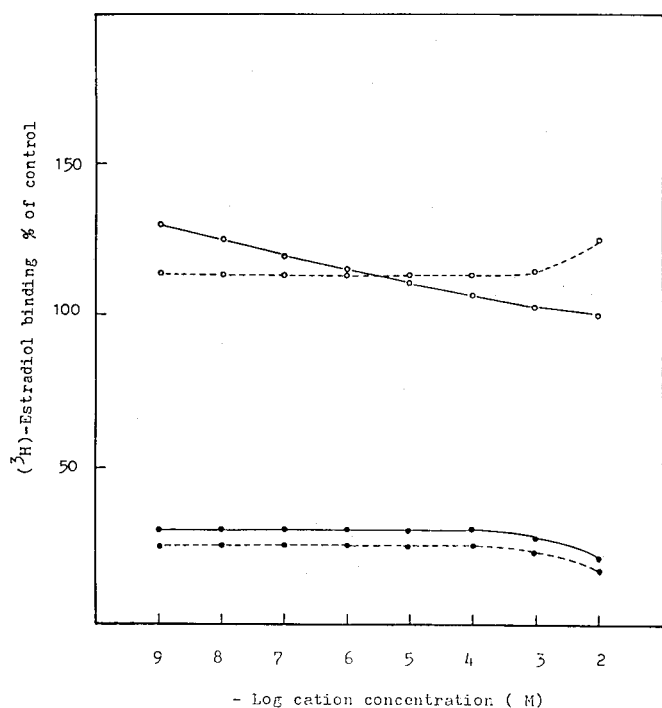


Fig. 1: Effect of cations on (³H)-estradiol binding to cytosol (•) and nuclear (o) receptors from breast cancer tissue [(-) Ca²⁺ and (...) Mg²⁺], (Case D).

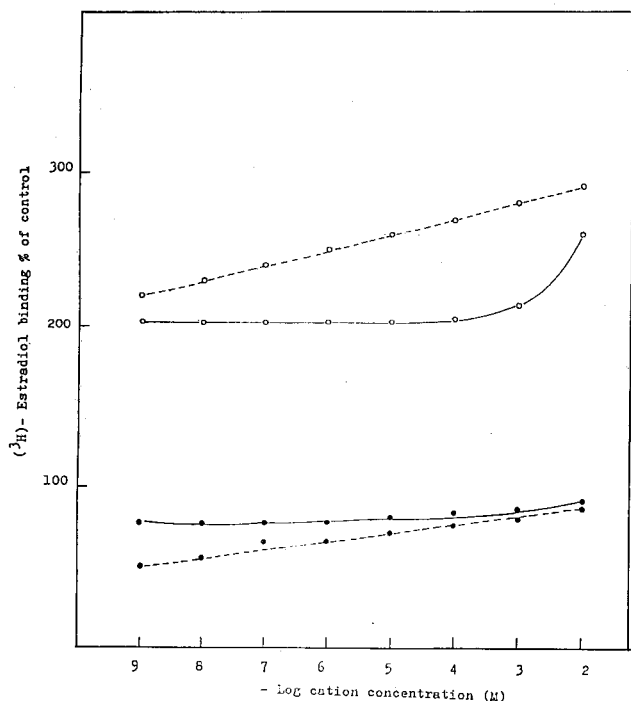


Fig. 3: Effect of cations on (³H)-estradiol binding to cytosol (•) and nuclear (o) receptors from breast cancer tissue [(-) Ca²⁺ and (...) Mg²⁺], (Case B).

The results for all breast carcinoma and uterus cytosols are presented in Table 1. The results demonstrate that:

- (a) cytosol of case D was indistinguishable from normal human uterus (Figs. 1 and 2) with regard to their binding affinity towards estradiol at various concentrations;
- (b) cytosols of cases A and C that contain specific estradiol

receptor show a wide range of affinities for estradiol with a variation in the number of available binding sites (Table 1); (c) case B lack the presence of receptors in the cytosol (receptor negative), meanwhile it does not lack the presence of nuclear receptors. Accordingly, the estimation of estradiol receptors in the cytosol fractions of the breast carcinoma tissue may not be a true index of the functional activity of estradiol receptor. So, in order to predict the endocrine nature of the tumor more accurately, estimation of nuclear estrogen receptors should prove to be more valuable.

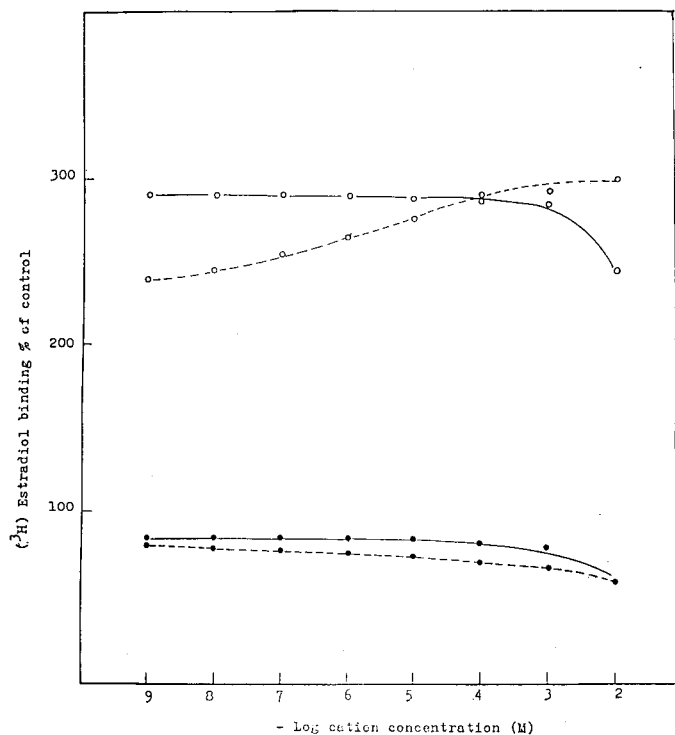


Fig. 4: Effect of cations on (^3H) -estradiol binding to cytosol (\bullet) and nuclear (\circ) receptors from breast cancer tissue [(-) Ca^{2+} and (...) Mg^{2+}], (Case A).

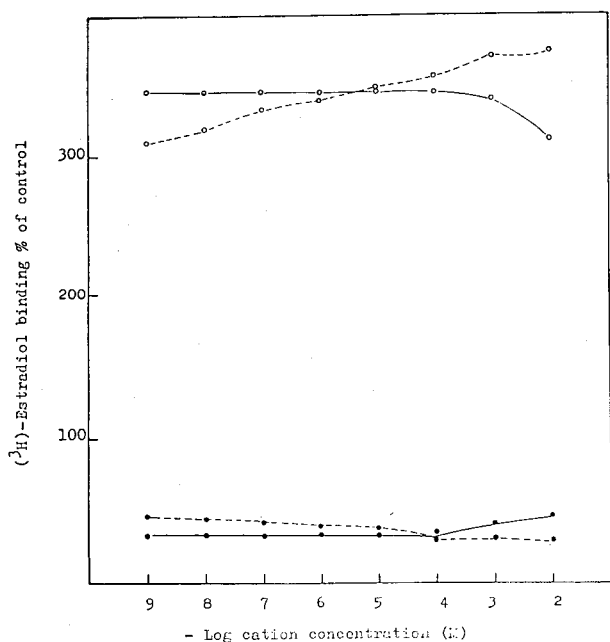


Fig. 5: Effect of cations on (^3H) -estradiol binding to cytosol (\bullet) and nuclear (\circ) receptors from breast cancer tissue [(-) Ca^{2+} and (...) Mg^{2+}], (Case C).

Table 1
The value of K_d and B_{max} from the Scatchard plot of the cytosol and nuclear pellet of the different breast cancer cases compared with the human uterus (reference tissue)

Case	Cytosol		Nuclear Pellet	
	Dissociation Constant (K_d)(M)	Maximum binding site(s) (B_{max}) (pmoles/mg protein)	Dissociation Constant (K_d)(M)	Maximum binding site(s) (B_{max}) (pmoles/mg protein)
Uterus	0.648×10^{-9}	4.1×10^{-3}	1.27×10^{-9}	0.089
A	a) 3.75×10^{-9}	0.045	3.2×10^{-9}	2.48
	b) 1.10×10^{-9}	0.025		
B*	-ve	-ve	2.42×10^{-9}	0.28
C	a) 1.44×10^{-9}	0.062	2.42×10^{-9}	2.49
	b) 0.40×10^{-9}	0.017		
D	0.46×10^{-9}	0.017	3.43×10^{-9}	0.181

a) & b) types of specific estradiol receptors.

* Recurrent tumor (recurrent infiltrating duct carcinoma mass on the chest wall on the chest wall, 2.5 years after radical mastectomy).

Also, Table 1 represent the K_d , and B_{max} values of the nuclear receptors. The maximum binding sites (B_{max}) were always higher in the nuclear pellet than in the cytosol whereas, the affinity range change slightly.

It is also observed that the dissociation constant (K_d) of estradiol receptors was high in the cytosols and nuclear pellets of breast carcinoma tissues compared with that from human uterus indicating a higher affinity in the latter when compared with that of the former. These results are in agreement with that of Singh *et al.* (1978) for cytosolic receptors.

It is also observed that the maximum binding sites in the breast carcinoma were higher than that in the uterus. The cytosolic receptors demonstrate also high variability in their characteristic and lower affinity for estradiol in case of breast carcinoma tissue as compared to the receptors of normal uterus (Okulicz, 1987). These changes in the receptor affinity could be one of the causes of malignant transformation of breast tissue, which still remains to be determined.

From Tables 1 and 2, the presence of Ca^{2+} the a uterine nuclear preparation caused no change in the maximum binding sites of the receptors. This was accomplished with decrease in the affinity of the receptor for estradiol. The concentration of Ca^{2+} required for this effect was 10^{-5} and 10^{-3}M . In typical mammalian cell, the concentration of free calcium is about 0.1 micromolar. On the other hand, the same result was obtained in presence of Mg^{2+} with the same concentrations. This suggests that the divalent cations altered the binding properties of the nuclear estrogen receptor in the nuclei of the human uterus. However, in the rat uterus, Ca^{2+} had no effect on the binding affinity of the receptor for estradiol but it causes aggregation of the receptors (Schoenberg and Clark, 1980).

The major effect of cations was demonstrated on the nuclear estrogen receptor in human mammary tumor, cases A, C and D. It is clear from the data in Tables 1 and 2 that the low cation concentration in this assay ultimately increases the

Table 2

The effect of different concentrations of divalent cations on the values of K_d and B_{max} from the Scatchard plot of the nuclear pellets of the different breast cancer cases compared with the uterus (reference tissues).

Case	Ca ²⁺ Concentrations				Mg ²⁺ Concentrations			
	10 ⁻³ M		10 ⁻⁵ M		10 ⁻³ M		10 ⁻⁵ M	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
Uterus	3.31x10 ⁻⁹	0.072	5.46x10 ⁻⁹	0.092	1.66x10 ⁻⁹	0.031	6.00x10 ⁻⁹	0.159
A	0.219x10 ⁻⁹	2.261	0.65x10 ⁻⁹	4.199	0.796x10 ⁻⁹	1.93	1.93x10 ⁻⁹	6.30
B	-ve	-ve	-ve	-ve	2.50x10 ⁻⁹	0.155	1.12x10 ⁻⁹	0.070
C	1.66x10 ⁻⁹	2.381	1.948x10 ⁻⁹	4.484	0.75x10 ⁻⁹	0.19	1.98x10 ⁻⁹	3.135
D	0.43x10 ⁻⁹	0.198	2.31x10 ⁻⁹	0.565	1.1x10 ⁻⁹	0.23	2.29x10 ⁻⁹	0.518

number of binding sites and their affinities compared with measured in the absence of cations. This effect was not clearly observed with the high cation concentrations (10⁻³M). It may be due to apparent aggregation of the receptors, which altered their physical association state. The data showed the same trend in all tumor tissues case B which was recurrent. This may indicate that the recurrent tumor possesses different affinity and behaviour towards the action of these cations. Accordingly, the degree of the therapeutic efficiency would face some sort of controversy because of such biochemical changes in the receptor entity. The study of the effect of different concentrations (from 10⁻⁹ to 10⁻² M) of divalent cations on (³H-estradiol binding revealed a mixed activation of the nuclear receptor binding of all studied tumor cases (see Figures 1, 3, 4, and 5). On the other hand, no obvious changes in the nuclear pellet of the uterus was detected (Fig. 2). This result may prove the differences in receptor nature between normal and cancerous tissues, and may suggest the importance of divalent cations especially Ca²⁺ in the binding of the hormone to its receptor in the breast cancer tissue. This effect which is lacked in the cytosol may give an idea that Ca²⁺ could be important in the translocation of estrogen receptor complexes to the nucleus rather than activation of proteolytic enzymes (Schneider and Dao, 1977; Rasmussen, 1986).

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