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Modulation of the intracellular calcium level in mammalian cells caused by 17β-estradiol, different phytoestrogens and the anti-estrogen ICI 182780

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Abstract

In previous investigations it was shown that the synthetic estrogen diethylstilbestrol (DES) induces a rise of the intracellular calcium level ($[Ca^{2+}]_i$) in C6 rat glioma cells [P. Tas, H. Stopper, K. Koschel, D. Schiffmann, Influence of the carcinogenic oestrogen diethylstilboestrol on the intracellular calcium level in C6 rat glioma cells. Toxic. In vitro 5 (1991) 463–465] which is accompanied by changes of the arrangement of the cytoskeleton. In the present study, we compared the induction of these effects in COS (monkey kidney cells) lacking estrogen receptors (ER) with those in RUCA-I (rat endometrial carcinoma) cells containing ER. The $[Ca^{2+}]_i$ in RUCA-I and COS cells following 17β-estradiol (ES), genistein (GEN), daidzein (DZ) and coumestrol (CES) treatment was analyzed. A significant increase of $[Ca^{2+}]_i$ induced by all compounds was observed in RUCA-I cells. No effects were detected in COS cells after ES and GEN treatment. The anti-estrogen ICI 182780 completely blocked the ES-and GEN-induced rise of $[Ca^{2+}]_i$. Dose and time dependencies of changes of calcium levels were analyzed and a biphasic response could be observed. The actin staining showed disintegrated stress fibers in RUCA-I cells. The degree of the observed effects correlates with the known estrogenicity of the applied compounds (DES > ES > GEN). It remains to be elucidated whether or not the effects observed are mediated by the "classic" genomic estrogen receptor pathway or by alternate nongenomic or receptor-independent pathways.

1. Introduction

Environmental compounds which exert estrogenic activities (environmental estrogens) have the potential to interfere with the endocrine system of humans and animals, thereby causing severe developmental, reproductive and oncologic disorders. The elucidation of many of these well documented observations is rather difficult, because neither the relative potency nor the molecular mechanisms of these compounds are known. The importance of an endometrial derived model system stems from the issue specifity of estrogen action which is well documented for the difference of mammary carcinoma cells and endometrial adenocarcinoma cells in their response to tamoxifen (anti-estrogen) treatment [1]. The number of suitable cell culture models to study hormonal functions in endometrial cancer cells is rather limited, because either endometrial tumor cell lines of human and rodent endometrial adenocarcinoma do not express steroid hormone receptors or if they express e.g. estrogen receptor (ER) in vitro, they respond to estrogens and anti-estrogens rather marginally [2]. Schütze et al. [3] described the establishment and characterization of two ER-positive rat endometrial adenocarcinoma cell

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lines: RUCA-I and RUCA-II. If cultured under standard cell culture conditions, with plastic as substrate and a medium containing charcoal stripped fetal calf serum (DCC-FCS), both cell lines expressed the ER [4].

Some environmental estrogens trigger their estrogenic response by replacing endogenous estrogens in the signaling pathway. Mostly, this means direct binding to the estrogen receptor. Most of the xenoestrogens, however, exhibit only weak binding affinity to the ER if compared to natural hormones. 17β-estradiol, the natural ligand of the ER, exhibits extensive binding to carrier proteins of the serum, whereas the synthetic hormone DES (diethylstilboestrol), is void of this affinity. This means at an equivalent blood concentration, more DES enters the cell than estradiol. In effect, DES is a functionally more efficient estrogen than the natural hormone [5]. In investigations of Tas et al. [6], it was shown that DES further causes a dose-dependent elevation of the cytoplasmatic calcium concentration in C6 rat glioma cells.

In the study presented, we investigated the effects of different estrogens, the natural estrogen: 17β-estradiol, and the phytoestrogens: daidzein, genistein and coumestrol, on the intracellular calcium level in RUCA-I cells. To investigate whether the influence of estrogens on $[Ca^{2+}]_i$ is dependent on the estrogen receptor, we compared ER-positive RUCA-I and ER-negative COS cells (monkey kidney cells). Additionally, we studied the actin filament structure in estrogen treated RUCA-I cells.

2. Materials and methods

2.1. Cell cultures

RUCA-I cells were established as described previously [3]. COS (kidney, SV40 transformed, African green monkey, fibroblast like cell line) cells were purchased from ATCC (USA). Prior to experimental use, RUCA-I and COS cells were precultured for one passage in DMEM/F12 medium without phenol red containing 10% FCS (Gibco, Karlsruhe, Germany) and for two passages in DMEM/F12 containing 5% dextran-coated charcoal (DCC) treated FCS. The experiments were carried out in the presence of serum-free defined medium (SFDM). The SFDM was composed of DMEM/F12 and contained additionally 2 μg/ml insulin, 4 mM glutamine, 40 μg/ml transferrin, 10 nM hydrocortisone, 20 nM sodium selenite, and 1 μg/ml putrescine. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

2.2. Hormonal treatment

For hormonal treatment, the natural estrogen 17β-estradiol (10 nM–1 μM), the anti-estrogen ICI 182780 (500 nM, Schering, Germany) and the phytoestrogens genistein (1–10 μM, Sigma, Germany), daidzein (1–10 μM, Roth, Germany) and coumestrol (1 μM, Fluka, Germany) were used. The synthetic estrogen diethylstilbestrol (DES, 20 μM, Sigma, Germany) was used as positive control for actin staining experiments. The relative binding affinity of ICI 182780 to the estrogen receptor is approx. 5-fold weaker than that of estradiol [7].

2.3. Measurement of intracellular calcium concentration

Treated and untreated RUCA-I and COS cells were incubated in PBS/CMF (phosphate buffered saline/ calcium and magnesium free; 30 min, 37°C) containing the calcium indicator fluo-3 AM (final concentration: 40 μM; Molecular Probes Inc., USA). Cells were washed with PBS and plated on Petriperm dishes. For each specimen 10 different frames were recorded at 488 nm excitation wave length and 515 nm emission wave length (long path) using a confocal laser scanning microscope (Noran Odyssey XL on a Nikon Diaphot 300). The $[Ca^{2+}]_i$ of individual cells was measured by determining the fluorescence intensity with the Intervision Software (Noran) on a Silicon Graphics Workstation. For measurements of the mean fluorescence intensity, 3–5 experiments with 20–50 cells each were taken in respect.

2.4. Actin staining

Treated and untreated RUCA-I cells were fixed in paraformaldehyde (3%) for 15 min and treated with TBS + 0.1% Triton X-100 for permeabilization of the cell membrane. The cells were incubated with a blocking solution (1% bovine serum albumin and 2% normal goat serum in TBS) for 5 min at room temperature and subsequently incubated with rhodamine/ phalloidin (final concentration: 0.5 μM; Sigma, Germany) for 15 min. After washing with TBS, the cells were mounted with antifade and the actin stress fibers were observed directly using a fluorescence microscope.

3. Results

Treatment of RUCA-I rat endometrial adenocarcinoma cells with 17β-estradiol or genistein induced an increase in $[Ca^{2+}]_i$. This response is biphasic with a first maximum of increase of $[Ca^{2+}]_i$ after 2 min and a second burst after 12 to 18 h of treatment (Fig. 1).

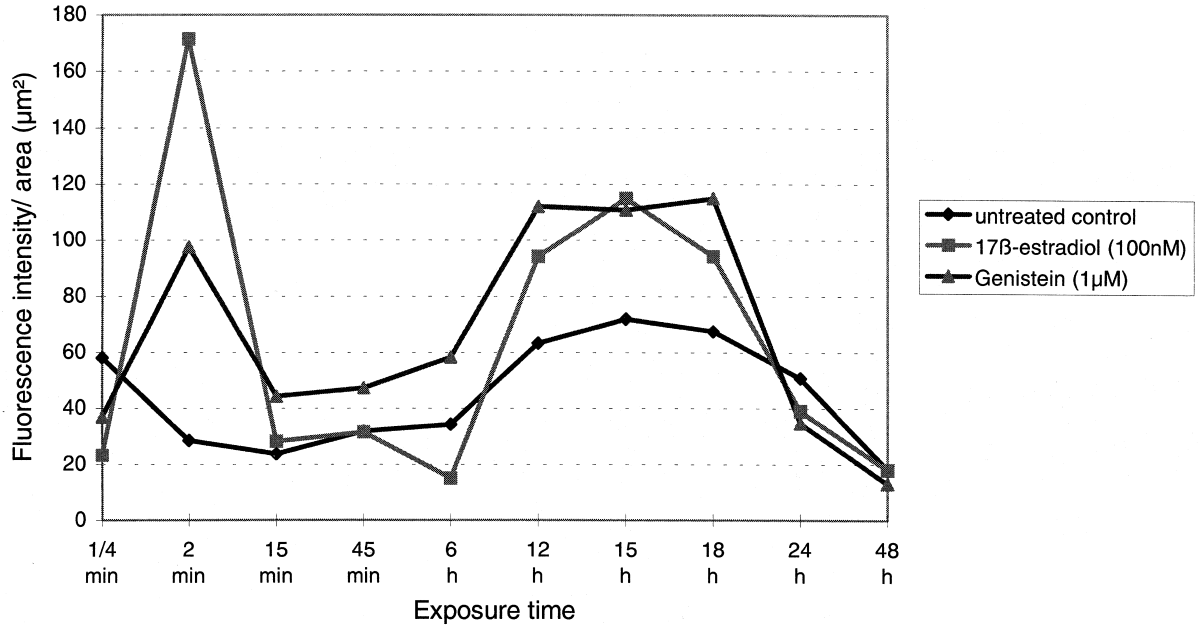

Fig. 1. Determination of the fluorescence intensity of estrogen-, genistein-treated and untreated RUCA-I cells in dependence on exposure time (SD $\leq$ 18.5%)

The increases of intracellular calcium concentrations after 2 min are 6-fold higher after ES-treatment and 3-fold higher after GEN-treatment in comparison to control levels. The second burst shows a longer lasting signal (6 h) and is just 1.5-fold higher after ES- or GEN-treatment in comparison to the untreated control (Fig. 1). The dose responses for the natural estrogen 17β-estradiol and the phytoestrogen genistein are not identical: The maximal increase of $[Ca^{2+}]_i$ was reached at 100 nM estradiol and 10 µM genistein respectively (Fig. 2). At higher ES-concentrations (1 µM–10 µM) the $[Ca^{2+}]_i$ decreased again. A comparison of different phytoestrogens (daidzein 1 µM, genistein 1 µM, coumestrol 1 µM) with estradiol (100 nM) showed that the phytoestrogens (in particular daidzein) are also effective inducers of an increased $[Ca^{2+}]_i$ at physiologically relevant concentrations (Fig. 3). The differences between treated and untreated cells are statistically significant in all cases (ES, GEN, DZ: $p \leq 0.001$; CES: $p \leq 0.01$; Fisher exact test (1-tailed), Fig. 3). These results do not correlate with the relative binding affinities of coumestrol, genistein, and daidzein to the ER (Table 1).

Two different cell systems were compared to distinguish between estrogen-receptor-dependent (RUCA-I cells) and -independent (COS cells) effects. An

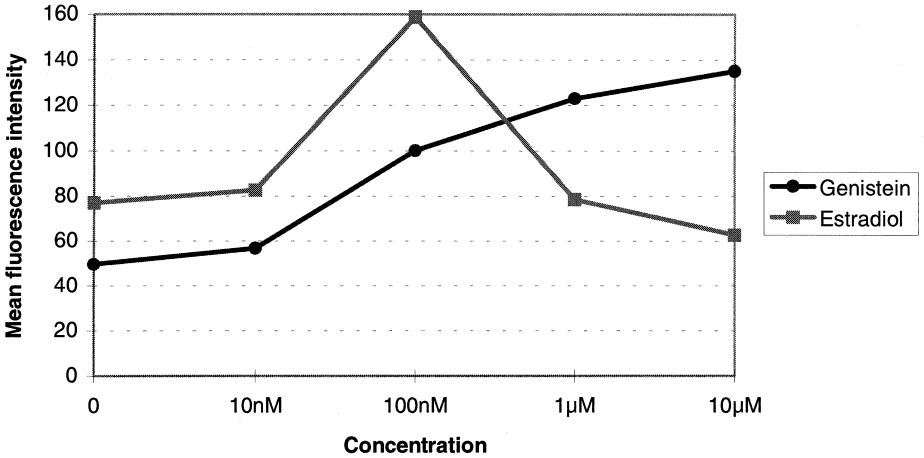

Fig. 2. Measurements of intracellular calcium concentrations in RUCA-I cells following concentration dependent treatment with estradiol and genistein (18 h exposure time). The cells were loaded with Fluo-3 AM for determining the fluorescence intensity. (SD $\leq$ 17.7%)

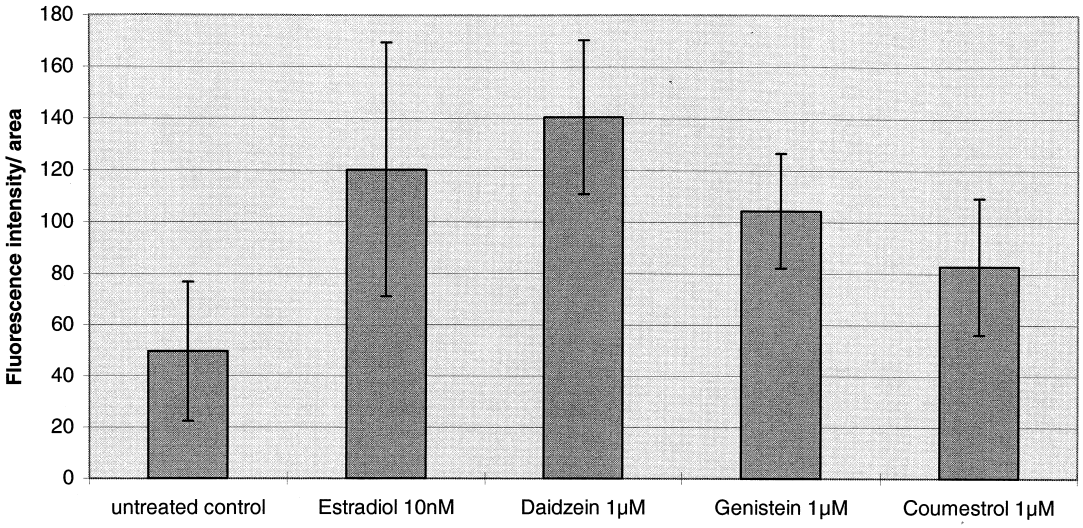

Fig. 3. Fluo-3AM fluorescence level of estrogen induced $[Ca^{2+}]_i$ increase in RUCA-I cells. Exposure time was 18 h in all cases.

Table 1
Relative binding affinities of coumestrol, genistein and daidzein compared to 17β-estradiol (Data taken from Hopert et al. [14] for comparison)

Evaluation method	Coumestrol (%)	Genistein (%)	Daidzein (%)
y-Axis[a]	2.15±1.00	0.82±0.48	0.015±0.01
Slope	1.88±0.84	0.92±0.40	0.015±0.01

[a] Binding affinities were evaluated by using the slope or the intersection of *y*-axis of the Scatchard analysis [14]. Both evaluation methods gave similar results.

increase of the $[Ca^{2+}]_i$ was observed in RUCA-I but not in COS cells following exposure to estradiol. The increase of $[Ca^{2+}]_i$ after 18 h of exposure was suppressed as a response to a combined treatment of RUCA-I cells with estradiol (10 nM) and ICI 182780 (50 nM, Fig. 4). The increase of $[Ca^{2+}]_i$ in ES-treated RUCA-I cells is statistically significant ($p \leq 0.001$) in comparison to the untreated control and to ES-treated COS-cells. The results of the combined treatment (ES + ICI) are not significantly different in comparison to control cells (Fig. 4). Comparable results were obtained after exposure of RUCA-I and COS cells to genistein and genistein + ICI (Fig. 5). The observed increase of $[Ca^{2+}]_i$ in GEN-treated RUCA-I cells is

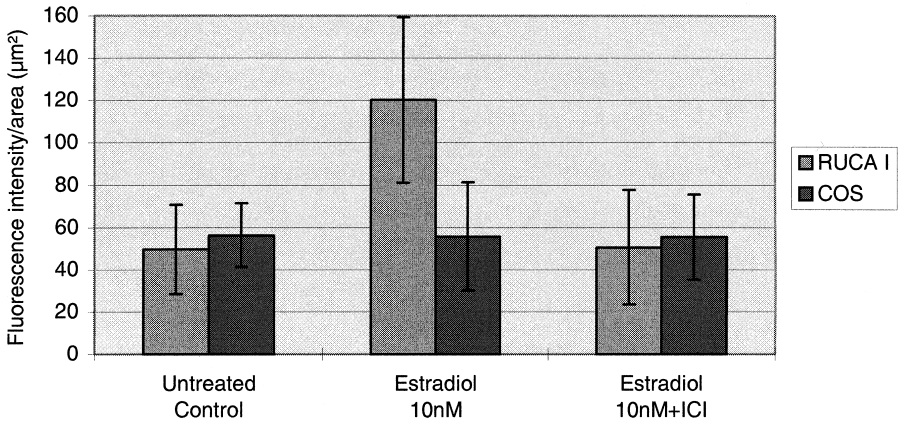

Fig. 4. Effects of incubation of RUCA I- and COS cells with 17β-estradiol (10 nM, 18 h) and 17β-estradiol (10nM, 18 h) + ICI 182780 (50 nM, 18 h) on the intracellular Ca^{2+}-concentration. The cells were incubated with Fluo-3 AM and the $[Ca^{2+}]_i$ of individual cells was measured by the fluorescence intensity with a confocal laser scanning microscope.

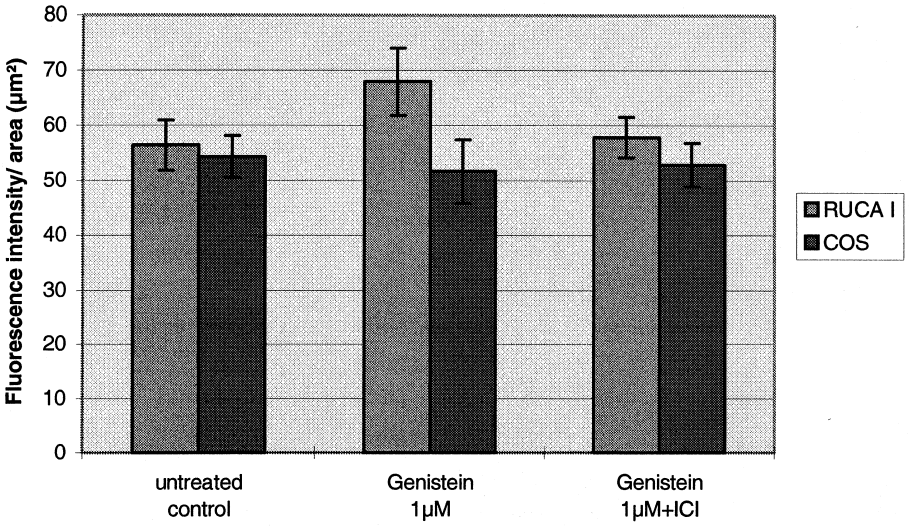

Fig. 5. Measurement of intracellular calcium concentrations in RUCA-I and COS cells following treatment of cells with genistein (1 µM, 20 h) and genistein (1 µM, 20 h) + ICI 182780 (5 µM, 20 h).

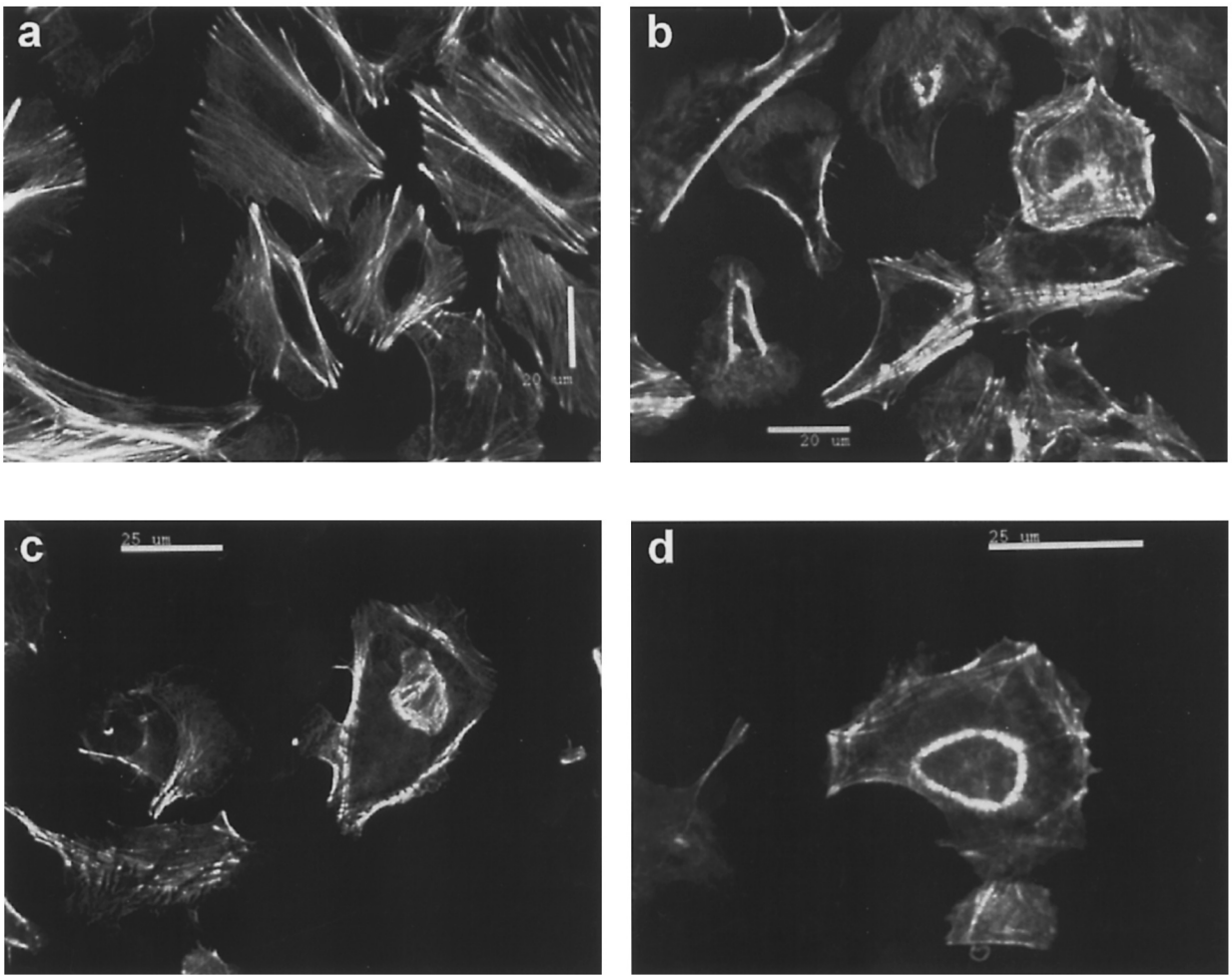

Fig. 6. Results of actin staining of RUCA-I cells following treatment with different estrogens: (a) untreated cells, (b) genistein treated (10 µM, 18 h) cells show slightly affected actin filaments, (c) stronger damaged actin filaments after treatment of cells with estradiol (100 nM, 18 h), (d) DES treated cells (20 µM, 18 h) were used as positive control. Cell shrinkage and depolymerization of stress fibers are observed.

Table 2
Measurement of intracellular calcium concentration in RUCA-Icells after 17β-estradiol- (100 nM) and a combined 17β-estradiol- (100 nM) and ICI 182780 (500 nM) treatment. Exposure time was 2 min. Measurements were carried out with a confocal laser scanning microscope

	Relative mean fluorescence intensity±S.D.
untreated control	47.7± 0.7
17β-estradiol (100 nM)	225.2±33.9
17β-estradiol (100 nM) + ICI 182780 (500 nM)	58.9±12.8

weaker than in ES-treated RUCA-I cells ($p \leq 0.05$). The combined treatment also did not reveal significant differences (Fig. 5). The first maximum of increase of $[Ca^{2+}]_i$ in ES-treated RUCA-I cells after an exposure time of 2 min can also be suppressed by ICI-treatment (Table 2). The basal level of $[Ca^{2+}]_i$ in these cell types was approximately 100 nM.

The actin staining revealed that the phytoestrogen genistein induces just a slightly change of the cell shape. Arrangement and morphology of the actin stress fibers is not affected in contrast to treatment of cells with estradiol and DES (Fig. 6). Estradiol treated RUCA-I cells showed a marked change of the cell shape, cell shrinkage and depolymerized actin stress fibers. The strongest effects were observed following treatment of RUCA-I cells with DES (Fig. 6).

4. Discussion

Tas et al. [8] reported that the aneuploidy-inducing carcinogenic estrogen diethylstilboestrol (DES) causes a dose-dependent elevation of the cytoplasmatic Ca^{2+}concentration in C6 rat glioma cells. In our present investigations, the main interest was focused on the induction of such effects in ER+ and ER− cell lines in vitro caused by 17β-estradiol and different phytoestrogens.

It is known that the $[Ca^{2+}]_i$ is a critical factor in mitosis and a considerable number of tumor promotors appear to be selective activators of the Ca^{2+}-phospholipid-sensitive protein kinase C (PKC). The physiological activators of this enzyme are diacylglycerols, which are produced by the breakdown of membrane lipids in response to extracellular signals [9]. Fujimoto et al. [10] reported that estrogens (estradiol) also activate PKC in endometrial cancer cells (ER +) and fibroblasts derived from human uterine endometrium (ER +). In contrast, tamoxifen (antiestrogen) inhibits PKC via oxidative stress [11]. The cell-signalling pathway involves PKC, phospholipase C, IP3-induced calcium mobilization or Ca^{2+}-channels. Doolan and Harvey [12] reported on an increased $[Ca^{2+}]_i$ via activation of PKC by steroid hormones in rat colonic epithelial cells. Yokoshiki et al. [13] investigated the influence of genistein and daidzein on L-type Ca^{+} channels in rat ventricular cells. Both, the tyrosine kinase inhibitor genistein and its inactive analog daidzein inhibit Ca^{+} currents in a dose-dependent manner. Genistein was less effective than daidzein. This may be either due to the difference in estrogen receptor α binding affinity [14] (Ruca-I cells do not contain the estrogen receptor β) or due to secondary effects of genistein mediated by tyrosine kinase. The action of daidzein may be mediated rather by an effect further downstream in the signalling pathway than by direct interaction with the receptor. However, this hypothesis requires more support by experimental data.

Morley et al. [15] investigated the effects of steroids (estradiol 17α, -17β, estrone, estriol and different androgens) on the $[Ca^{2+}]_i$ in chicken granulosa cells. In contrast to our investigations, these authors observed an immediate (less than 5 s) 4- to 8-fold increase in $[Ca^{2+}]_i$ after addition of 100 nM 17β-estradiol. Similar results were obtained in pig granulosa cells. The differences in time course of intracellular calcium changes obtained in granulosa cells of chicken or pigs and in endometrial adenocarcinoma cells of rats can be explained by activation of different cell surface receptors (the conventional gene-stimulating nuclear estrogen receptor is slow acting). The above authors also found that the estrogen-triggered $[Ca^{2+}]_i$ surge was not affected by incubating of cells with tamoxifen (10 μM). This also shows that different receptors were activated.

In our investigations genistein induced a prolonged calcium elevation in RUCA-I cells at higher doses compared to estradiol. This may be due to the fact that the relative binding affinity of genistein to the ER is about 100-fold weaker than that of estradiol. That means that 100-fold higher concentrations of GEN are necessary to induce comparable effects to ES. Daidzein binds even with a lower affinity to the estrogen receptor than GEN. Despite this fact, DZ appears to be the most potent inducer for the increase of $[Ca^{2+}]_i$ whereas both compounds induce the same level of

complement C3 gene expression in RUCA-I cells, if cells are treated at 100-fold higher concentrations as compared to estradiol [14]. DZ binds with a 50-fold lower affinity to the ER of RUCA-I cells than GEN, and has a roughly 5000-fold lower affinity than ES [14]. Despite this fact on the level of regulation of the gene expression, both, DZ and GEN, to the same extend induce an increase in complement C3 expression. Conversely, DZ is the most potent inducer of increased $[Ca^{2+}]_i$-levels. This means GEN and DZ are equally potent in terms of stimulation of ER-dependent gene expression despite significantly different relative binding affinities. Therefore, it is tempting to speculate that this high Ca^{2+}-effect induced by DZ compensates for lower binding affinity and leads to an equally potent gene regulatory effect, if compared to GEN.

The time course of GEN and ES action on the increase of $[Ca^{2+}]_i$-levels is nearly identical and it seems to be connected to the cell cycle. In untreated cells, the $[Ca^{2+}]_i$ is elevated e.g. during mitosis. It might be possible that estrogens are able to increase the $[Ca^{2+}]_i$ because of their mitogenic activity.

Several discrete Ca^{2+} pools seem to exist in most mammalian cells. Ca^{2+} signalling mediated by the intracellular messenger inositol, 1,4,5-triphosphate involves Ca^{2+} release from one or more discrete Ca^{2+} pools within the cell [16]. In investigations of Morley et al. [15] the 17β-estradiol-induced $[Ca^{2+}]_i$ spike was also not affected by incubating the cells in calcium-free medium. These findings are in accordance with our results. It can be concluded that the intracellular calcium rise is caused by a calcium release from intracellular stores. It remains to be elucidated if the estrogen-mediated Ca^{2+} rise is caused by release from Ca^{2+} stores of the endoplasmatic reticulum or from a mitochondrial source. Recent studies by Picotto et al. [17] have provided evidence for nuclear estrogen receptor-mediated calcium transport in intestinal mucosal cells. These authors induced an intestinal cell calcium influx by 17β-estradiol via the cAMP messenger system.

The suppression of the estradiol-induced elevation of the calcium level after 2 min and 18 h of exposure by the antiestrogen ICI 182780 in RUCA-I cells indicates that these types of response are receptor mediated.

Changes of the $[Ca^{2+}]_i$ can be connected with disturbances of the actin cytoskeleton [18]. DES is known to alter cytoskeletal components, including the organization of actin stress fibers in C6 rat glioma cells. Evidence exists which shows that cell morphology is intimately linked to levels of intracellular free calcium and that DES increases such levels [19]. The observed changes in stress fiber organization in our investigations induced by genistein, estradiol and DES (positive control) correlate with the estrogenicity and the calcium elevation induced by these compounds. These experiments were not carried out with unphysiologically high estrogen concentrations. We conclude that an endocrine effect was observed. In earlier investigations Schiffmann et al. [20] found similar effects in SHE cells. Therefore, at present, the question remains open to what extent these changes are mediated by estrogen receptors and whether these effects are genomic or nongenomic.

In summary, our investigations have shown that 17β-estradiol and different phytoestrogens induce a significant elevation of the $[Ca^{2+}]_i$ in RUCA-I cells (ER +), but not in COS cells (ER−). This ER dependent effect can be blocked by a combined estrogen- and ICI-treatment. The time course of estrogen activity measured by $[Ca^{2+}]_i$ revealed a biphasic response. The relative binding affinity of the phytoestrogens is about 100-fold weaker than that of estradiol, but the observed effects are similar. Changes of actin filament structure correlate with the estrogenicity of the applied estrogens.

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