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KERATINIZATION OF RAT VAGINAL EPITHELIUM—V. MODULATION OF INTRACELLULAR CALCIUM BY ESTRADIOL

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Summary—Changes in the calcium levels under the influence of estradiol were investigated in rat vaginal epithelial cells (VEC). After single estradiol injection, the immature rats showed 1.5-fold increase in Ca^{2+} levels within 15 min when compared to control animals. Progesterone priming brought calcium levels well below control values throughout the experimental period (up to 12 h). Ca^{2+} levels in serum did not show any appreciable change. Localization of calcium in VEC with electron microscopy showed aggregates of calcium oxalate on the inner nuclear membrane, nucleolus, mitochondria and keratohyaline granules. After 15 min of estradiol priming, maximum electron density was seen on all these cell organelles mentioned above, however, by 30 min the electron density was reduced considerably and did not increase during the experimental period (up to 12 h).

INTRODUCTION

During the differentiation of vaginal epithelial cells many events take place; some of these are known to be regulated directly or indirectly by the circulating levels of ovarian steroid hormones [1, 2]. The hormone estradiol is known to enter through the plasma membrane of the target cell. Physiochemical changes that occur in the membrane during the entry of estradiol are not precisely known; however, Reddy *et al.* [3] have shown that during the estrus phase of the estrous cycle, the plasma membrane becomes more fluid when compared to the diestrus phase. Membrane fluidity is known to affect permeability to various metabolites [4, 5].

We have observed that transglutaminase activity in vaginal tissue of immature or ovariectomized rats is increased 1.5–2-fold in estradiol-primed animals [6]. This enzyme plays an important role in cross-linking of keratins during keratinization of vaginal epithelial cells. This enzyme is known to be calcium-dependent [6, 7].

Extracellular calcium is a critical regulator of the growth and differentiation of normal rat vaginal cells [8] and mouse skin cells [9] in culture. When vaginal cells are cultured in a medium containing low calcium concentration (0.05 mM), they proliferate rapidly and form monolayer cultures [8]. When fetal calf serum and estradiol are added to the medium, cell proliferation decreases; cells begin to enlarge, stratify and cornify (produce rigid cross-linked envelopes) and then slough-off from the culture dishes as mature or fully differentiated cells. We studied the effect of estradiol on calcium levels in the vaginal epithelial cells.

We have thus estimated the total cell calcium levels in vaginal epithelial cells of immature estradiol-primed rats at various time intervals and have also monitored the Ca^{2+} levels after progesterone priming. Besides this, we have studied calcium localization after priming the rats with estradiol for various time points, using electron microscopy.

EXPERIMENTAL

Preparation of animals

Immature (30 day old) female Wistar rats housed at 25 ± 2°C with 12 h light and 12 h dark periods were used. The animals were given food and water *ad libitum*. Immature rats received a pharmacological dose of estradiol-17β (10 μg/100 g body wt) or progesterone (10 μg/100 g body wt) in propanediol i.p. The control rats received equivalent volume of propanediol (vehicle).

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Estimation of calcium

The control and hormone-primed immature rats were killed at various time points by cervical dislocation, their vaginae were excised quickly and the epithelial cells were scraped from the tissue with the help of a glass slide. The pooled vaginal epithelial cells from 10 animals in each group were homogenized. An aliquot from each homogenate was used for protein estimation, and the rest was dissolved in a mixture of nitric acid and perchloric acid (1:1, v/v) as described by Marrella and Milanino [10]. The samples were heated to near dryness and suitably diluted with deionised glass distilled water. The clear solution was used for the estimation of calcium in direct current plasma atomic emission spectrometer (Spectraspan V) using 393.3 and 445.4 nm as the emission lines. The values for calcium were essentially similar at both wavelengths. Reagent blanks were run with each set of digested samples. Spleen, representing a non-target tissue and serum (carrier of estradiol) were collected for calcium estimation from these animals. Spleen was processed as described above, whereas serum was used as such for calcium estimation after suitable dilutions. Lowry's method [11] was used to estimate proteins. Calcium values were calculated as μg per mg protein and expressed as percent of control.

Localization of calcium

Vaginae of 30 day old rats primed with estradiol for various time points were perfused with sodium oxalate solution (10 mM sodium oxalate in 140 mM potassium chloride) for 10 min [12]. After rinsing once in water, the vaginae were fixed in buffered glutaraldehyde and osmium tetroxide and processed as described by Gupta *et al.* [2]. Sections were examined without staining in uranyl acetate and lead citrate in JEOL 100 CX electron microscope using objective aperture (20 μm diameter).

Hormone assay

Estradiol levels in serum were estimated by radioimmunoassay technique. Control and estradiol-primed rats were bled before killing and serum was separated. Steroids were extracted from the serum by using solvent ether (recovery 86.3% $\pm$ 3.6). Estradiol was measured in serum using radioimmunoassay kits supplied by World Health Organisation, London, England. The interassay and intraassay coefficient of variation at standard containing 12.5 pg/ml estradiol were 2.49 and 1.72 respectively. Hormone concentrations are expressed as mean $\pm$ SE.

RESULTS

The estradiol levels in the serum of experimental and control rats are given in Fig. 1. In control animals, the levels of estradiol were 58.42 $\pm$ 4.15 pg/ml. There was a rise in estradiol levels in the serum within 15 min reaching a peak at 924.85 $\pm$ 103 pg/ml. The hormone levels started declining and were maintained around control levels (59.50 $\pm$ 8.39 pg/ml) from 6 h onwards.

The changes in calcium levels were studied under the influence of estradiol in immature rats at different time intervals in target tissues such as vaginal epithelial cells, non-target tissues such as spleen and the carrier fluid, serum. There was a marked increase in Ca^{2+} influx in vaginal epithelial cells in animals as early as 10 min after the injection of estradiol in the peritoneal cavity. The Ca^{2+} level reached a peak within 10–15 min and then gradually decreased to below the control value by 30 min and remained low up to 12 h after the treatment (Fig. 2). Progesterone treatment, on the other hand, did not trigger the influx of Ca^{2+} in the cells; levels of Ca^{2+} remained lower than the control throughout the experimental period (up to 12 h).

The effect of estradiol treatment on the Ca^{2+} levels in the spleen and the serum are given in Fig. 3. The Ca^{2+} levels in the serum remained almost closer to control values throughout the experimental period in the estradiol-primed animals. There is a substantial decrease in the levels of Ca^{2+} in the spleen of estradiol-primed animals during early time points, however, the

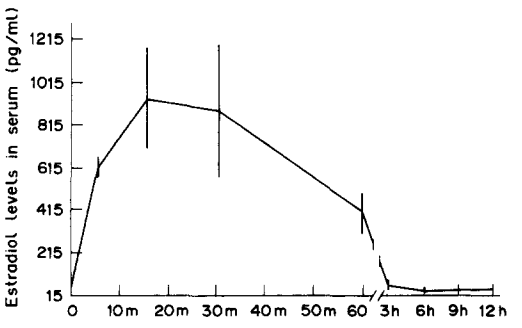

Fig. 1. Quantitative analysis of estradiol in the serum from rats primed with the hormone. Each point on graph represents mean $\pm$ SD of estradiol levels in 4 animals. Data is representative of two similar experiments.

Ca^{2+} levels remained constant throughout the experimental period thereafter (Fig. 3).

Calcium was localized as aggregates of fine electron dense particles in transmission electron microscopy after immobilizing calcium as calcium oxalate by infusing sodium oxalate in the tissue. In both the primed and unprimed rat vaginal tissues, aggregates of calcium oxalate were seen deposited on the innernuclear membrane and the nucleolus in the nuclear compartment, and mitochondria and the keratohyaline granules in the cytoplasm (Fig. 4a–d). In the primed animals, however, these sites showed more electron density compared to preparation from unprimed animals (cf. Fig. 4a and c with b and d). This increased electron density was seen as early as 15 min after estradiol priming, however, after 30 min of priming the cellular structures mentioned above did not show any electron density (Fig. 4e and f).

The general electron density in the cells again increased by 6 h after priming but not to the extent as in the controls. No electron density was seen along the nuclear membrane (Fig. 5a and b); on the other hand, electron dense granules were seen deposited on keratohyaline granules, tonofilament bundles and other cytoplasmic organelles, 6 and 12 h after priming the animals. The electron density in vaginal epithelial cells fixed without pretreatment with sodium oxalate was much less (Fig. 5c) compared to primed as well as unprimed animals treated with sodium oxalate. However, vaginal epithelial cells from rats treated with estradiol for 30 min (Fig. 4e and f) showed the minimum electron density compared to all preparations mentioned above.

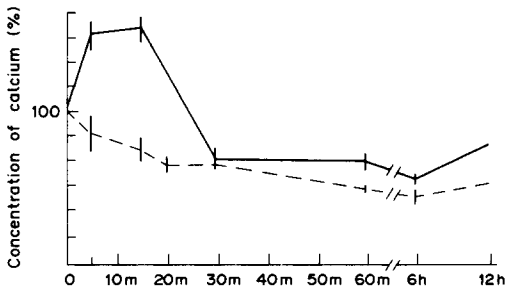

Fig. 2. Effect of estradiol (——) and progesterone (– – –) treatment on the Ca^{2+} levels of vaginal epithelial cells of immature rats. Each point on the graph represents the calcium level of pooled vaginal epithelial cells from 10 animals, each sample was analysed 3 times and average values were taken for calculation. The values are given as percentage of controls (mean ± SD).

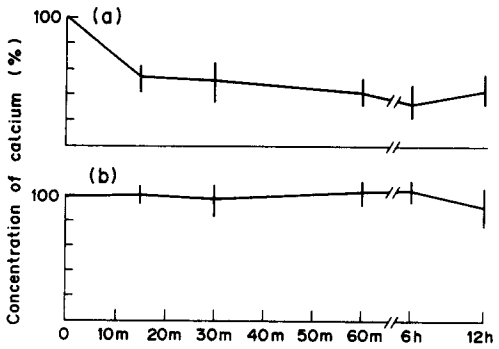

Fig. 3. Effect of estradiol on the Ca^{2+} levels of (a) spleen cells and (b) blood serum. Each sample was analysed 3 times and average values were taken for calculations. SD was computed from data on 5 animals for each time point (mean ± SD).

DISCUSSION

It is well known that keratinization of vaginal epithelial cells takes place in adult cycling rats during estrus phase of the estrous cycle. Changes similar to keratinization could be induced in immature rats, upon priming with estradiol [2, 13]. In cultures, the vaginal epithelial cells grow as a monolayer in low concentration of Ca^{2+} and estradiol. Addition of Ca^{2+} in the presence of estradiol results in the formation of clumps and sheath-like structures similar to the stratified epithelium, suggesting a definite role for Ca^{2+} in estradiol-induced differentiation of vaginal epithelium [14].

The present *in vivo* experiments also indicate that intraperitoneal injection of estradiol in immature rats increases estradiol levels in serum and concomitantly Ca^{2+} levels in vaginal epithelial cells also increase. Within 30 min Ca^{2+} levels come down well below control levels; however estradiol levels in serum is maintained up to 3 h. Intracellular localization of Ca^{2+} by electron microscopy confirmed the data obtained by atomic emission spectrometry.

Calcium is known to regulate many cellular processes [15, 16]. Calcium levels are maintained at the basal level in the cells most of the time; however, as the need arises, there is stimulation for Ca^{2+} influx. The present studies show that Ca^{2+} levels acutely increase within 15 min in vaginal epithelial cells after injection of estradiol in the peritoneal cavity of rats. Similar increase in Ca^{2+} levels are also reported in estradiol stimulated endometrial cells *in vitro* [17] and in progesterone-depleted animals [18]. Homo and Simon [19] have shown that hormones which induce Ca^{2+} uptake do so when they act as agonists. In the present experiments we have

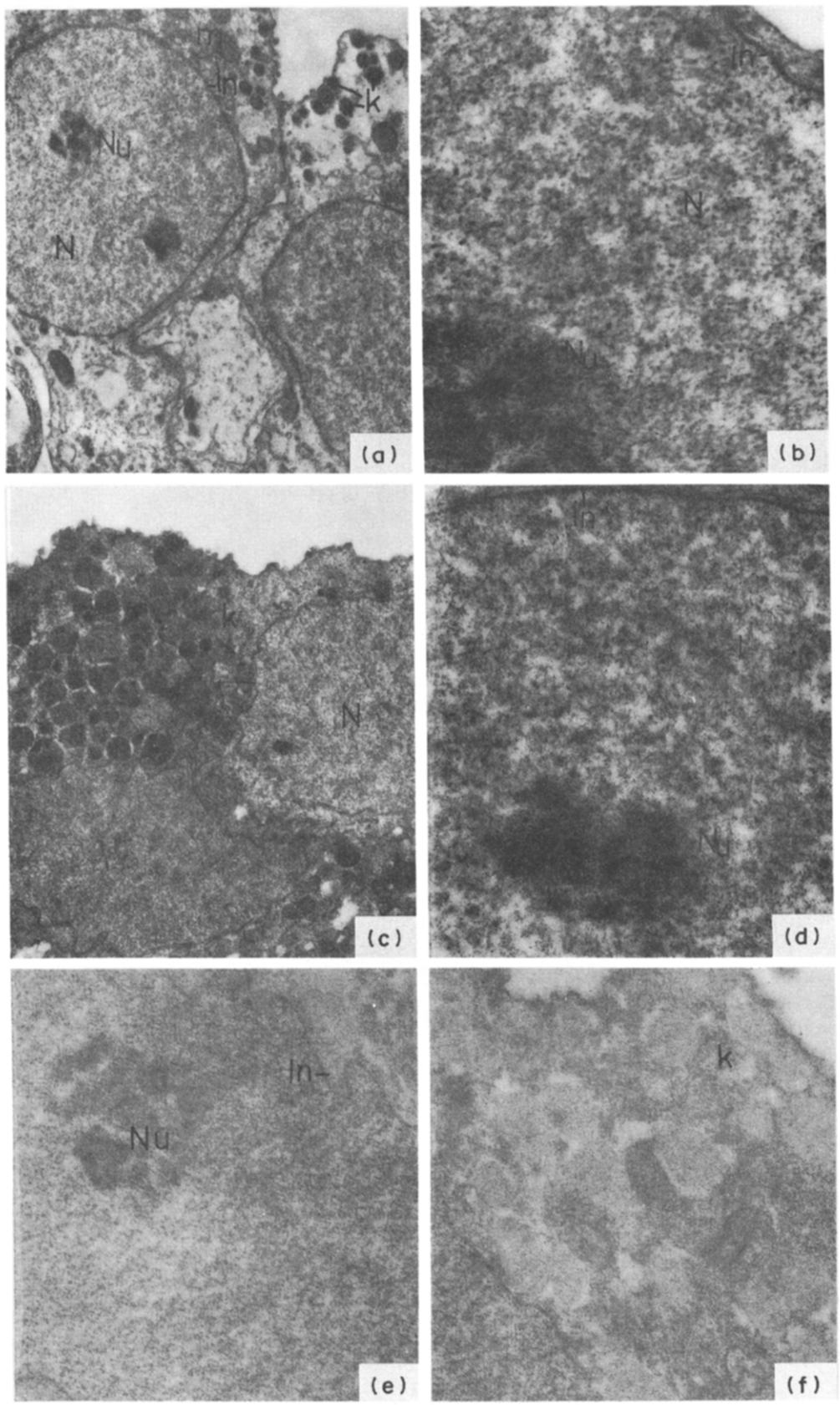

Fig. 4. (a and b) Electron dense fine granules of calcium oxalate are seen deposited on the inner nuclear membrane (In), the nucleolus (Nu) and the keratohyaline granules (k) mitochondria (m) of vaginal epithelial cells from unprimed 30-day-old rats. (Magnification (a) × 10,000; (b) × 16,000.) (c and d) Vaginal epithelial cells from rats primed with estradiol for 15 min. Overall electron density increases due to more deposition of calcium oxalate granules on the nucleolus and the innernuclear membrane and in the ground cytoplasm. (Magnification (c) × 10,000; (d) × 13,000.) (e and f) Vaginal epithelial cell from rats primed with estradiol for 30 min. Electron density is considerably reduced on the cell organelles and also in the ground cytoplasm. (Magification (e) × 10,000; (f) × 10,000.)

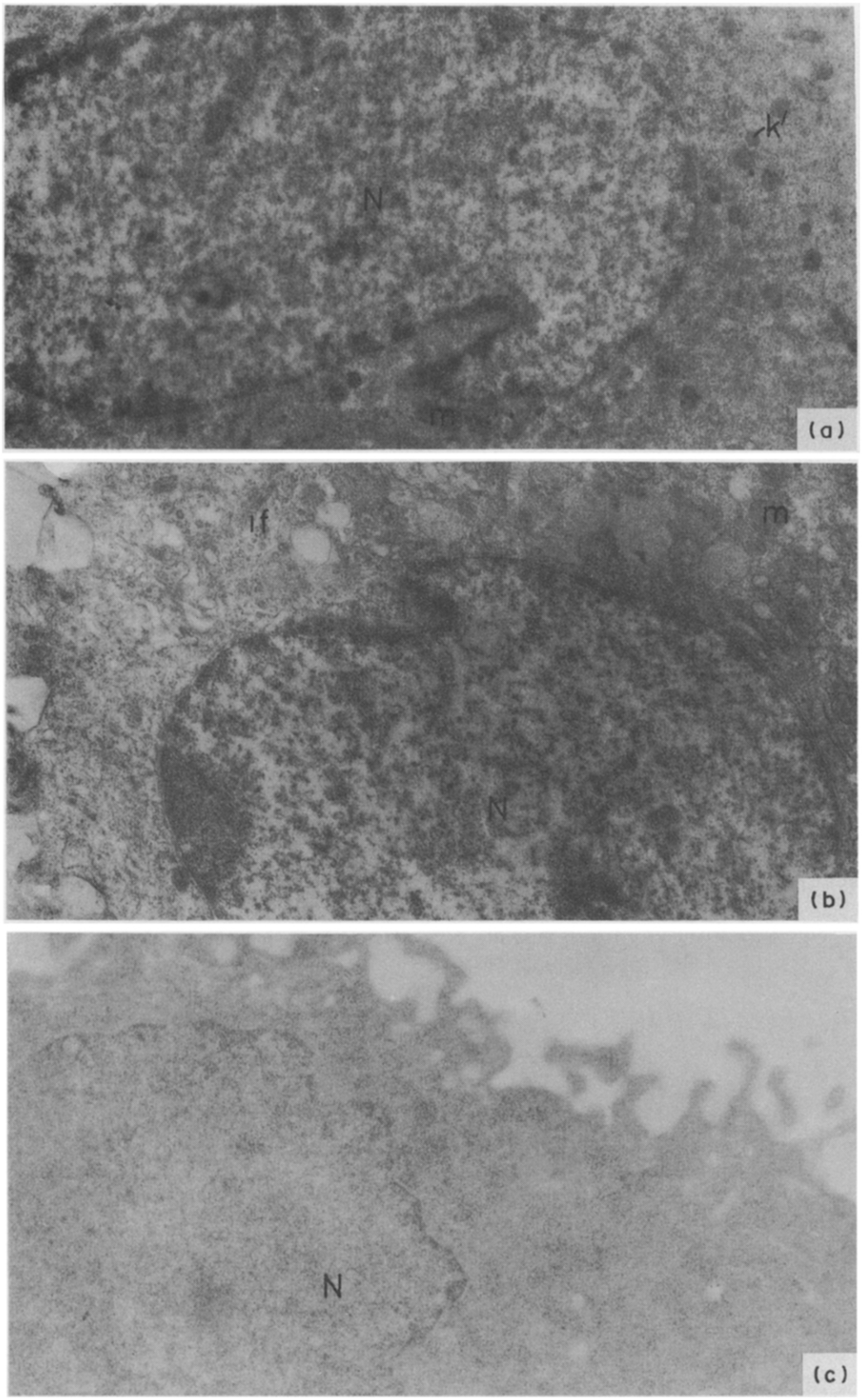

Fig. 5. (a) Vaginal epithelial cell from rat primed with estradiol for 6 h. Keratohyaline granules again show electron density (cf. Fig. 4e and f). Inner nuclear membrane (In), does not show any electron density. (Magnification $\times$ 13,000.) (b) Vaginal epithelial cell from rat primed with estradiol for 12 h. Distribution and intensity of electron dense material is similar to (a) however, cell cytoplasm in granule is more electron dense, intermediate filaments (if). (Magnification $\times$ 13,000.) (c) Epithelial cell from rat vagina without any treatment showing no electron density (cf. Fig. 4e and f). (Magnification $\times$ 13,000.)

shown that estradiol increases Ca^{2+} uptake in 30-day-old animals under controlled experimental conditions. Animals treated with progesterone always showed lower levels of Ca^{2+} than control animals. These observations go in line with the fact that progesterone produces effects opposite to that of estradiol in target cells as shown by many workers [20, 21].

The levels of Ca^{2+} in serum or non-target tissues show no significant change after the hormone treatment in the present study. Rasmussen [22] showed that concentration ratio (exterior to interior) of calcium across the cell membrane is 1×10^2–1×10^5. Therefore it is likely that small changes, in target cells where the concentration is very low, could not be monitored upon hormonal stimulation. The initial lowering of Ca^{2+} levels in the spleen due to estradiol treatment is difficult to explain.

While commenting on mechanism of Ca^{2+} uptake, Homo and Simon [19] mentioned that glucocorticoids and similar compounds increase membrane permeability to Ca^{2+}. In our earlier studies we have shown that membrane fluidity of vaginal epithelial cells is greater in estrus phase as compared to proestrus and diestrus. Membrane fluidity is known to affect the permeability of metabolites such as glucose [4], acetate [5], and some cations. A number of peptide hormones which alter Ca^{2+} concentration involve inositol triphosphate in Ca^{2+} mobilization [23]. Stojilkovic *et al.* [24] have suggested that calcium uptake in pituitary cells occurs either by voltage sensitive calcium channels (sensitive to membrane depolarization) or receptor operated Ca^{2+} channels (activated by specific agonist receptor interaction). Hormones, mitogens and antigens bring alterations in Ca^{2+} levels in their target cells by stimulating hydrolysis of membrane phosphoinositides, which is brought about by activation of phospholipase C by agonist receptor complex [25–28].

Hormones induce either Ca^{2+} oscillation [29] or an initial peak followed by gradual decay to a plateau in target cells [30]. In vaginal epithelial cells estradiol induces calcium peak at 15 min which decreases by 30 min. Calcium is known as second messenger in hormone signal transduction in systems where cAMP level are not influenced [31] by the hormone. A number of Ca^{2+}-dependent reactions are reported in estradiol induced vaginal epithelial cells, though the increased levels of Ca^{2+} were seen in the initial 15 min. Ca^{2+} levels were later on maintained at control levels.

Our earlier observation that the Ca^{2+}-dependent enzyme transglutaminase also showed increased activity after estradiol administration [6] impresses on the requirement of excess amount of Ca^{2+} in the cell during keratinization. It is possible that the increase in Ca^{2+} influx induced by estradiol-17β may also be responsible for the enhancement of transglutaminase activity.

Further, our preliminary observations (unpublished) on phosphorylation of keratin polypeptides from vaginal epithelial cells indicated the involvement of Ca^{2+} in this post-translational process. Phosphorylation of keratins takes place between 15 and 30 min in estradiol-treated cells. This time period matches well with the Ca^{2+} influx in vaginal cells. Calcium ionophore A 23187 also brings about the same effect on phosphorylation of keratin polypeptides.

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