Modulation of phosphorylation and dephosphorylation of keratin and other polypeptides by estradiol-17β in rat vaginal epithelium

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Phosphorylation of keratin polypeptides was studied by incubating vaginal tissues (removed from estradiol primed and unprimed 30-day-old rats) with $^{32}P_i$. Analysis by SDS-PAGE and autoradiography showed that on treatment with estradiol phosphorylation of 63 and 58 kDa keratin polypeptides increased 3- and 2-fold respectively. Phosphorylation was maximal after 30 min of estradiol priming and decreased thereafter. Phosphorylation of some non-keratin polypeptides (37, 34, 32 and 25 kDa) also showed time dependent variation. The results showed that estradiol can modulate phosphorylation–dephosphorylation of keratins and other polypeptides in rat vaginal epithelial cells.

Phosphorylation; Dephosphorylation; Estradiol; Keratin; Vaginal epithelium

1. INTRODUCTION

Steroid sex hormones exert their effects on various metabolic processes in target organs. Growth and terminal differentiation (keratinization) of rat vaginal epithelial cells (VEC) are regulated by estradiol [1–3]. VEC are characterized by the presence of keratins which form a dense, insoluble intracellular matrix during progressive differentiation of these cells [1,4]. The expression of actual number and size of keratin peptides depends on various factors such as type of cell [5], growth condition [6], stage of differentiation [1] and chemical modulators such as vitamin A [7], estrogen [8] and tumor promotors [9].

Keratins belong to the intermediate filament (IF) class of proteins and are prominently phosphoproteins [4,10]. Keratins get phosphorylated after being synthesized both in vivo and in vitro [11–14]. Gilmartin et al. [15] have shown that phosphorylation of IF is responsible for assembly and distribution of these filaments. Since the synthesis of keratins in VEC is regulated by estradiol, it is reasonable to expect that the functions performed by keratins in these cells should also be regulated by this hormone. To throw some light on this possibility, we have studied phosphorylation of keratins from rat VEC under the influence of estradiol-17β and found that phosphorylation of keratins is modulated by estradiol.

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Abbreviations: VEC, vaginal epithelial cells; IF, intermediate filament; DMEM, dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; CPM, counts per minute

2. MATERIALS AND METHODS

Randomly bred female Wistar rats were housed at constant temperature (25 ± 2°C) and 12 h light and dark cycle conditions. Vagina was dissected out from rats after 15, 30, 60, 120, 180 and 240 min of the peritoneal injection of estradiol-17β. Control rats received equivalent volume of the vehicle (70% ethanol).

For phosphorylation of vaginal cell proteins under the influence of estradiol, vagina was incubated in phosphate-free DMEM-containing [^{32}P]H_3PO_4 (10 μCi/ml) for 2 h at 37°C; after washings epithelial cells were scraped from the incubated tissue. The cells were suspended in 10 mM Tris-HCl containing 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM sodium vanadate [16,17] to prevent dephosphorylation of the proteins. To prevent proteolytic action, 0.4 mM PMSF and 1 μg/ml soyabean trypsin inhibitor were added to the buffer. The cells were homogenized and keratin fraction was isolated as described earlier [4]. Protein estimation was done by Lowry's method. Both, total homogenate and keratin-enriched fractions were analysed on SDS-PAGE. SDS gels were stained with Coomassie blue, dried and exposed by placing them in contact with X-ray film at −70°C for 24 h. Vaginal tissues were also incubated in calcium ionophore A23187 (38 nM) in DMEM for 1 and 2 min and then for incorporation of $^{32}P_i$ these were processed as described above. Radioactive counts for individual keratin polypeptide bands were recorded by cutting the bands from SDS gels and counted.

Some gels were treated with 1 N sodium hydroxide solution for 1 h at 56°C to digest alkali-labile phosphate [18]; after the alkali treatment the gels were dried and autoradiographed. Calcium levels in vaginal tissues after the treatment with estradiol for various time points were estimated in direct current plasma atomic emission spectrometer using the 393.3 and 445.4 nm as emission lines [19].

3. RESULTS AND DISCUSSION

The effect of estradiol on phosphorylation of proteins present in rat vaginal epithelial cells was studied by incubating vaginal tissue (removed from unprimed and estradiol primed rats) with $^{32}P_i$ in DMEM as described in section 2. Phosphorylated polypeptides showed differences in the level of $^{32}P_i$ incorporation (Fig. 1a). The changes in phosphorylation of 63 and 58 kDa polypep-

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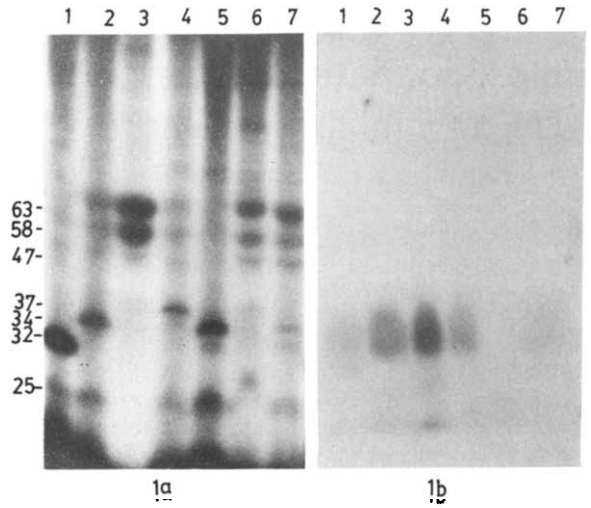

Fig. 1 (a) Autoradiogram of VEC homogenate on SDS-PAGE showing incorporation of $^{32}P_i$ in polypeptide bands in unprimed (lane 1), primed with estradiol-17β for 15 min (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5), 180 min (lane 6) and 240 min (lane 7). After priming with estradiol tissues were incubated with $^{32}P_i$ and processed further as described in section 2. The gel was exposed overnight at −70°C. (b) Autoradiogram of the alkali treated SDS-PAGE of the above preparations. Lanes 1, 2, 3, 4, 5, 6 and 7 are as above (Fig. 1A). The gel was exposed for 72 h at −70°C.

tides (which are perhaps keratin polypeptides) induced by estradiol were time dependent. Maximal phosphorylation was observed after 30 min of peritoneal injection of estradiol; phosphorylation of these polypeptides decreased after 30 min, showing the lowest level at 60 min. Phosphorylation of 63 and 58 kDa keratin polypeptides was increased 3- and 2-fold respectively after estradiol treatment (Figs 1a, 2b). Marked changes in the phosphorylation of some other polypeptides of 37, 34, 32 and 25 kDa were also observed (Fig. 1a). The most prominent phosphorylated polypeptide of 32 kDa was dephosphorylated rapidly within 15 min of estradiol injection. The 34 kDa polypeptide showed increased phosphorylation after 15 min of estradiol injection and then was dephosphorylated; rephosphorylation of this band was observed after 2 h of estradiol treatment (Fig. 1a). Time-dependent variation in the phosphorylation of 37 and 25 kDa polypeptides was also observed.

Keratin enriched fraction was isolated from $^{32}P_i$-labelled vaginal cell homogenate from control and estradiol treated animals. SDS-PAGE and autoradiography of these keratin enriched samples showed that 63, 58 and 47 kDa polypeptides are being phosphorylated (Fig. 3a,b). The 63, 58 and 47 kDa phosphorylated polypeptides correspond to keratin polypeptides on Coomassie blue stained gel of isolated keratin (Fig. 3a). The identity of other polypeptides whose phosphorylation is influenced by estradiol is not known.

Phosphorylation of keratins and other polypeptides (37, 34, 32 and 25 kDa) observed by us was alkali-labile (Fig. 1b), suggesting that Ser or Thr residues of keratins are being phosphorylated. However, alkali-stable phosphorylation of certain other polypeptides was observed under the influence of estradiol which also showed maximal levels after 30 min of estradiol injection (Fig. 1b). The increased phosphorylation of keratin

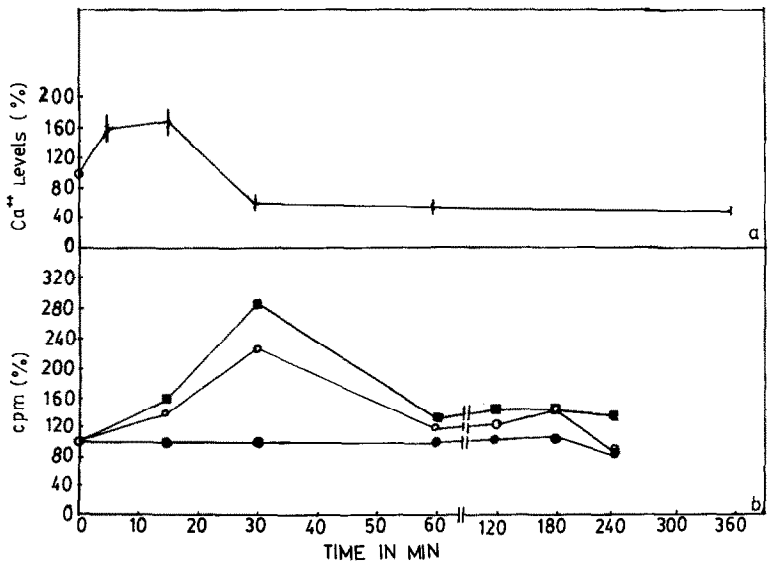

Fig. 2. (a) Ca^{2+} levels in VEC after estradiol treatment for various time points. Each point on the graph represents the Ca^{2+} level of pooled VEC from 5 rats. Each sample was analysed 3 times and average values were taken for calculation. The values are given as percentage of controls. Ca^{2+} was measured by atomic emission spectrometer at 393.3 and 445.4 nm emission lines. Both emission lines gave almost the same concentrations. (b) Incorporation of $^{32}P_i$ in polypeptide bands of 63, 58 and 47 kDa. The keratin enriched fraction after incorporation of $^{32}P_i$ was run on SDS gel and the $^{32}P_i$ incorporated polypeptide bands were sliced and counted in a Packard scintillation counter. The values are given as percentage of control (unprimed) rats. (■) 63; (○) 58 and (•) 47 kDa bands.

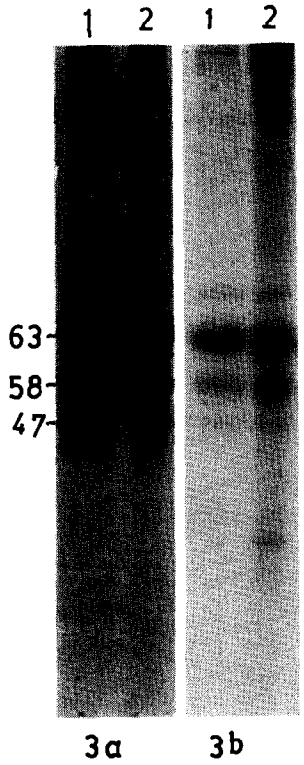

Fig. 3. (a) SDS gel of keratin enriched fractions from VEC. Lanes 1 and 2 represent keratin profiles of unprimed and primed animals. Coomassie blue stained preparation. (b) Autoradiogram of the same preparation. Lanes 1 and 2 show incorporation of $^{32}P_i$ in keratins in unprimed and primed rats. The gel was exposed overnight at −70°C.

polypeptides due to estradiol is not related to the increased synthesis of keratin polypeptides which do not occur at least until 6 h of estradiol treatment (figure not shown).

Phosphorylation of keratin polypeptides was maximal after 30 min of estradiol injection whereas phosphorylation of other polypeptides (mol. mass 37, 34, 32 and 25 kDa) was minimal at this stage. This complex pattern of phosphorylation of one set and dephosphorylation of the other set of polypeptides under the influence of estradiol suggests that, (A) it is unlikely to be due to direct effect of estradiol on protein kinase or phosphoprotein phosphatase activity, and (B), the activity of more than one protein kinase and/or phosphoprotein phosphatase may be influenced by estradiol. The nature of protein kinases and/or phosphoprotein phosphatases whose activities may be indirectly regulated by estradiol is not known.

Since Ca^{2+} is known to regulate activity of some protein kinases and phosphoprotein phosphatases, the possible involvement of Ca^{2+} in mediating the estradiol-induced changes in phosphorylation was explored. Estradiol increases uptake of Ca^{2+}; maximal level of Ca^{2+} was observed after 15 min of estradiol injection (Fig. 2a). These results raise the possibility that Ca^{2+} may be involved in mediating some of the effects of estradiol in rat VEC.

Phosphorylation of keratin polypeptides from epidermis has been studied extensively [12], however, estradiol-modulated phosphorylation of keratin polypeptides has not previously been reported. Two-fold increase in phosphorylation was recorded after the norepinephrine treatment in vimentin [20]. Fey et al. [21] have shown that the half-life of the phosphate is 13 min for keratins and 11 min for vimentin and therefore on phosphorylation these proteins behave differently. In our earlier studies [4] we have shown the structural differences and also differences in phosphorus contents between epidermal and VEC keratins. Recently, Yeagle et al. [22] have shown that the keratin subunits have different specific activities, probably due to a different number of phosphorylation sites. Further, they have shown with the help of NMR spectrometry that due to phosphorylation a portion, most likely the variable region of the molecule, keratins, becomes relatively rigid. Extensive cross-linking [23] and more rigidity of keratins [22] due to phosphorylation under the influence of estradiol would favour keratinization of VEC.

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