THE INFLUENCE OF LEUPEPTIN, MOLYBDATE AND CALCIUM IONS ON ESTROGEN RECEPTOR STABILITY

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1. Introduction

Proteolytic cleavage of 9–10 S estrogen receptors by endogenous enzymes present in extracts of human breast carcinoma and myometrium is inhibited by leupeptin and molybdate [1,2]. Both agents appear to protect the receptor to a similar degree [1,2]. Leupeptin is a known inhibitor of serine and thiol proteases [3], while molybdate has been reported to effectively restrict phosphatase activity [4]. Studies with breast tumour cytosol [1] and calf receptor extracts [5] have implicated respectively, plasminogen activators and a Ca^{2+}-activated protease in estrogen receptor modification. This paper describes the Ca^{2+} mediated prevention of estrogen receptor stabilization by leupeptin and molybdate and provides evidence that the mode of molybdate inhibition of hydrolytic enzyme activity may be partly due to its ability to remove endogenous Ca^{2+} by precipitation.

2. Materials and methods

Human myometrial tissue, frozen in liquid nitrogen, was pulverized for 40 s in a microdismembrator (Braun, Melsungen). The tissue powder was suspended in ice-cold $T_{10}D_1$ buffer (tissue:buffer, 1:2) containing 10 mM Tris–HCl (Sigma, St Louis MO) and 1 mM dithiothreitol (Calbiochem, Carlingford) at pH 7.4 or $P_{10}D_1$ buffer containing 10 mM potassium dihydrogen orthophosphate (Ajax Chemicals, Sydney) and 1 mM dithiothreitol at pH 7.4. Cytosol was isolated as in [6]. Estrogen receptors in the cytosols were manipulated by the addition of varying concentrations of sodium molybdate (Mallinckrodt, St Louis MO), leupeptin (Peptide Institute, Protein Research Foundation, Minoh-Shi, Osaka), EDTA-ethylenediamine tetraacetic acid disodium salt (British Drug Houses, Boronia) and $CaCl_2$ (Ajax Chemicals) to the buffer prior to tissue suspension or after cytosol had been isolated. Sodium azide (Ajax Chemicals, 0.02%, w/v) was present in all buffers. Prior to density gradient centrifugation estrogen receptors were labelled with 5 nM [^{3}H]estradiol-17β (54 Ci/mmol, Radiochemical Centre, Amersham) after incubation at 4°C for 2 h. Free and loosely bound labelled hormone was removed by treatment with the pellet from an equal volume of a dextran-coated charcoal suspension (1% charcoal, Norit A, Pfanstiehl; 0.1% dextran T_{40}, Pharmacia, Lane Cove in 10 mM Tris–HCl buffer (pH 7.4)) over 20 min at 4°C. Aliquots (200 μl) of the labelled extracts were layered on 20–50% (v/v) glycerol gradients prepared in $T_{10}D_1$ or $P_{10}D_1$ buffer. All gradients were centrifuged in an MSE-75 Ultracentrifuge at 145 000 × g for 16 h at 4°C using a titanium swing-out rotor. Aldolase (rabbit muscle, Serva, Heidelberg) and ovalbumin (Serva) were used as markers.

3. Results

The inclusion of Ca^{2+}, in cytosols and density gradients, completely negated the stabilizing influence of leupeptin and molybdate on the 9 S estrogen receptor from human myometrial cytosols and produced a 4 S binding entity exclusively (fig.1). This effect of Ca^{2+} was initially observed with 10 mM leupeptin in Tris-buffered cytosols containing 100 mM Ca^{2+}, but was subsequently shown to occur with as low as Ca^{2+} 5 mM (fig.1).

When cytosol was preincubated with 50 mM molybdate or leupeptin and then made 10 mM with respect

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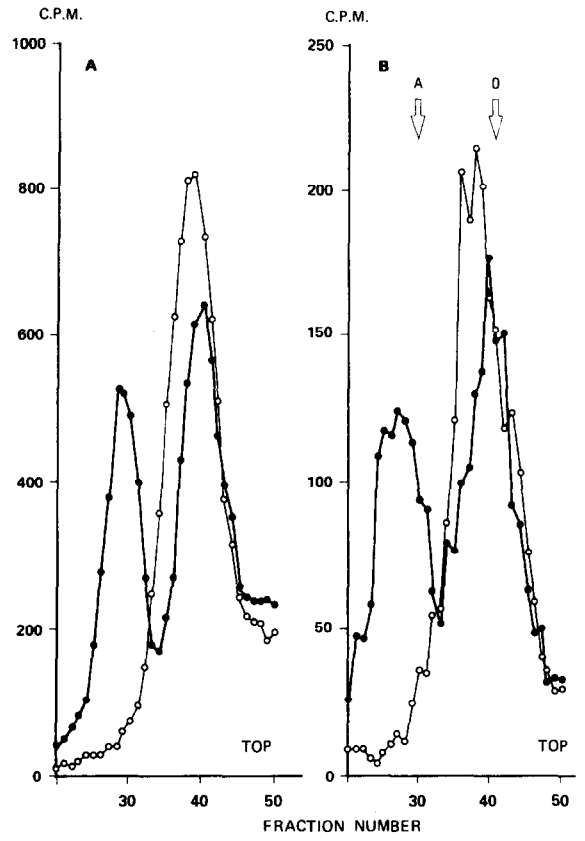

Fig.1. Sedimentation analysis of estrogen receptors in the presence of protease inhibitors, with or without excess Ca^{2+}. (A) Cytosols from the same tissue powder in $T_{10}D_1$ buffer containing 10 mM molybdate, with (o——o) or without (•——•) 5 mM $CaCl_2$, were labelled with [^{3}H]estradiol and subjected to glycerol gradient centrifugation in $T_{10}D_1$ buffer containing 5 mM $CaCl_2$ and $T_{10}D_1$ buffer, respectively. (B) Experiments with 10 mM leupeptin were carried out in the same way. Sedimentation markers: (A) aldolase (7.9 S) and (O) ovalbumin (3.6 S).

to Ca^{2+} the extent of 9–4 S receptor conversion was markedly decreased (fig.2). The inhibition was most pronounced with molybdate where 77% of receptor-bound [^{3}H]estradiol in the 9 S region was retained after Ca^{2+} addition (fig.2a). In the corresponding experiment with leupeptin, carried out on a different segment of the same tissue, the 9 S receptor in the leupeptin plus Ca^{2+} cytosol was stabilized to 48% of the leupeptin only control (fig.2b). A considerable decrease in total receptor-bound [^{3}H]estradiol was observed in the gradients from cytosols containing leupeptin compared to the unprotected extract, to which Ca^{2+} alone had been added (fig.2b). A similar decrease with molybdate was not apparent (fig.2a). From fig.1,2 it is evident that although 9 S receptor

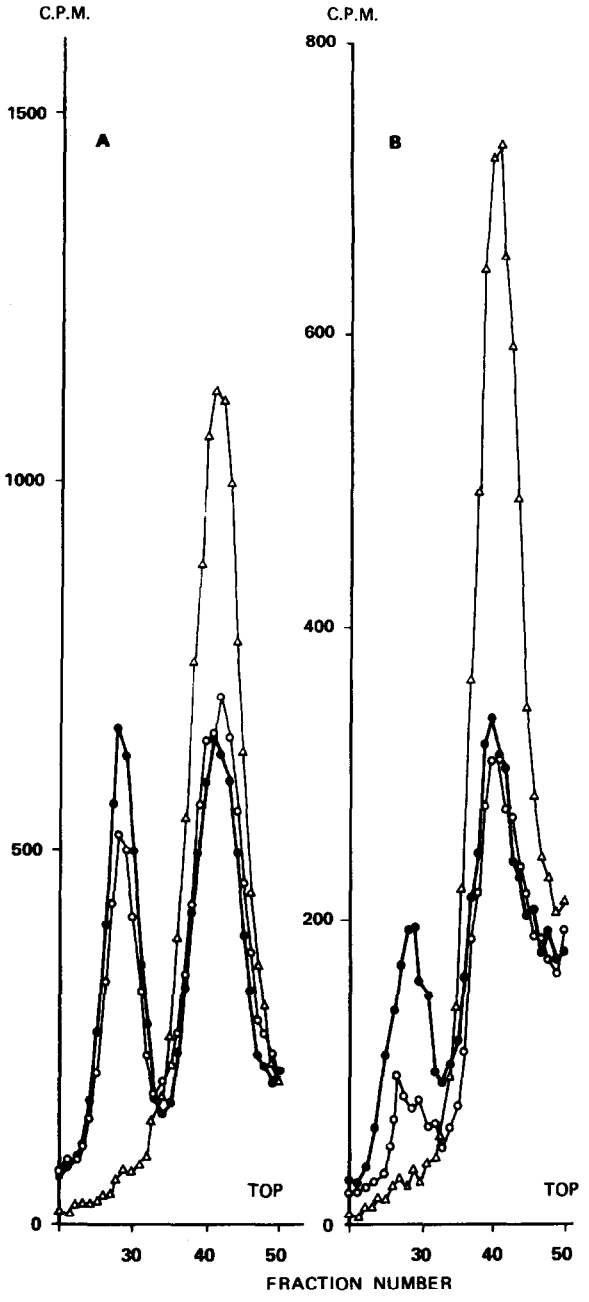

Fig.2. The influence of exogenous Ca^{2+} on the sedimentation profile of estrogen receptors pre-stabilized by molybdate and leupeptin. (A) Molybdate (50 mM) was added to an aliquot of freshly prepared myometrial cytosol in $T_{10}D_1$ buffer. After 15 min at 4°C $CaCl_2$ (10 mM) was added to the molybdate free cytosol and a portion of the cytosol containing the inhibitor. The resulting cytosols in $T_{10}D_1$ with 10 mM Ca^{2+} (△——△), $T_{10}D_1$ with 50 mM molybdate (•——•) and $T_{10}D_1$ with 50 mM molybdate and 10 mM Ca^{2+} (o——o) were labelled with [^{3}H]estradiol and subjected to glycerol gradient centrifugation in $T_{10}D_1$ buffer. (B) Experiments with 50 mM leupeptin, using a different segment of the tissue processed in (A) were done in the same way.

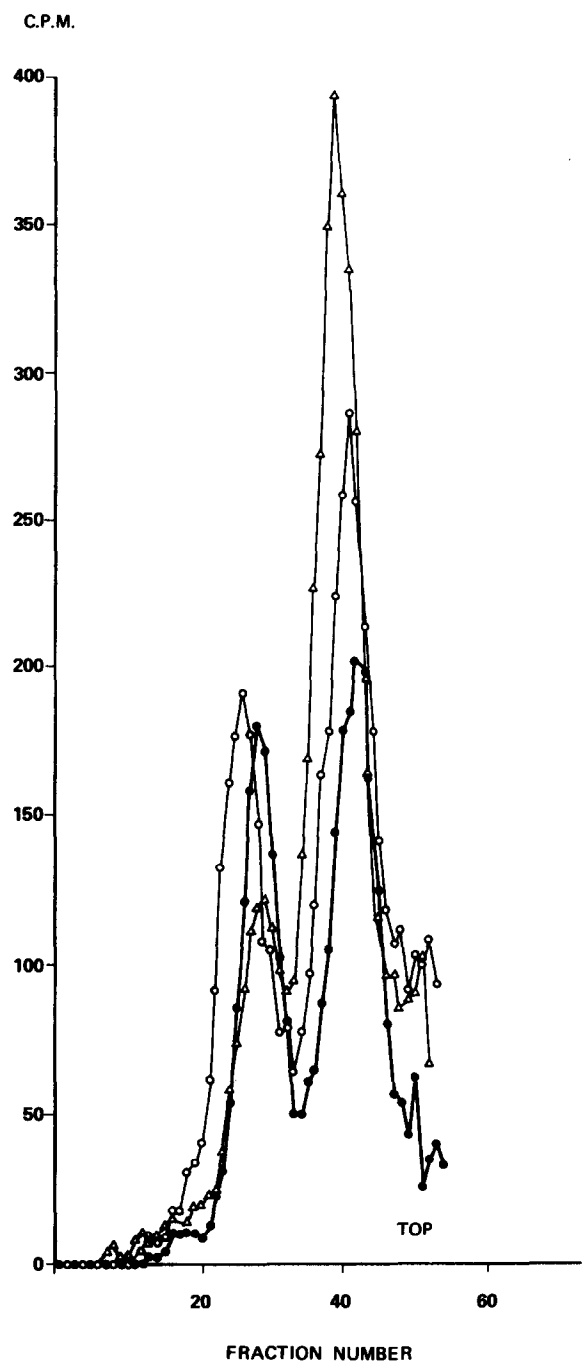

Fig.3. Comparison of sedimentation profiles of estrogen receptors from cytosols prepared in $P_{10}D_1$ (●——●), $T_{10}D_1$ (△——△) and $T_{10}D_1$ buffer containing 1.5 mM EDTA (○——○). The cytosols were prepared from the same tissue used for the experiments described in fig.2 and after labelling with [^{3}H]estradiol were subjected to glycerol gradient centrifugation in the corresponding buffers.

stability is enhanced by molybdate and leupeptin the smaller 4 S form is present in at least equal amount even at the relatively high inhibitor concentrations of 50 mM. The requirement of Ca^{2+} for 9–4 S receptor conversion and the rare occurrence of the large receptor form in Tris-buffered cytosols in the absence of protease inhibitors [1,2] suggested that there may be sufficient calcium of cellular origin to permit receptor modification. Molybdate readily precipitates Ca^{2+} from aqueous solution. This property and the ability of molybdate as against equal concentrations of leupeptin, to more effectively preserve the 9 S estrogen receptor indicate that molybdate may, to some extent, inhibit proteolytic activity by restricting the amount of Ca^{2+} available for calcium-dependent protease activation [5,7]. This mechanism of action for molybdate was supported experimentally. From the same tissue used to generate the results for fig.2 cytosols were prepared in $P_{10}D_1$, $T_{10}D_1$ and $T_{10}D_1$ buffer containing 1.5 mM EDTA. Density gradient centrifugation of each cytosol on gradients prepared in the corresponding buffer revealed that the peak height based 9 S:4 S ratio was highest for the phosphate buffer (0.89), followed by Tris-buffer with EDTA (0.67) and then $T_{10}D_1$ (0.31). Phosphate also removes Ca^{2+} by precipitation, while EDTA is a potent chelator of Ca^{2+} and other divalent cations. An adverse effect on receptor–[^{3}H]estradiol complex formation was noted with 10 mM EDTA in $T_{10}D_1$ buffer.

4. Discussion

In [5] the 8.6 S estrogen receptor in calf uterine cytosol was cleaved to a smaller 4 S unit by the 'receptor-transforming factor' – a protease which is activated by Ca^{2+} under conditions of high ionic strength. Ca^{2+}-Activated proteases have been described which catalyze the limited hydrolysis of the progesterone receptors in chick oviduct [8] and the glucocorticoid receptors in mouse mammary tumour [9] and rat kidney [10]. These results indicate that a similar Ca^{2+}-dependent enzyme exists in human myometrial cytosol and can modify the 9 S estrogen receptor after being activated by exogenous Ca^{2+} or endogenous Ca^{2+} in Tris-buffered myometrial homogenates. The larger receptor form is stabilized by molybdate and leupeptin [1,2], but if excess Ca^{2+} is present this protective effect is abolished. Receptor conversion catalyzed by exogenous Ca^{2+} was largely prevented

by preincubation with excess molybdate. Leupeptin was less able to counter the Ca^{2+}-activated protease, even at concentrations 5-times that of added Ca^{2+}. Contrary to this, in [9] higher M_r progesterone receptors were reported stabilized by 10 and 50 mM leupeptin in chick oviduct extracts containing 0.1 M $CaCl_2$. The same group [11] has also indicated that inhibition of the proteolytic breakdown of rat kidney glucocorticoid receptors by leupeptin is partially reversible. These comparisons suggest that there may be some differences in the nature of hydrolytic activity to which each receptor system has been exposed.

The decreased estradiol binding capacity of myometrial extracts to which 50 mM leupeptin had been added confirmed earlier observations with a number of human breast tumours [2]. Decreased binding levels with leupeptin were reported [1] and the loss attributed to foaming during homogenization and increased ionic strength. Our preparations were in hypotonic buffers and displayed little tendency to foam either with or without leupeptin. The consistent observation of an estradiol–receptor complex sedimenting in the 4 S region, even at high protease inhibitor concentration, may indicate that at least some 9 S receptor was modified by cytoplasmic proteases. However, the presence of a native 4 S estrogen receptor, distinct from the proteolysed form, is also a possibility. A native 4 S receptor which combines specifically with a cytoplasmic component to give an 8 S estradiol binding unit under hypotonic conditions has been described for cow uterus [12].

The increased stability of the 9 S estrogen receptor in cytosols containing molybdate, as well as in extracts containing phosphate and EDTA, suggests that molybdate prevents receptor modification, at least partly, by removing Ca^{2+} from solution, thus restricting Ca^{2+}-dependent protease activity. Phosphatase inhibition by molybdate [4] may also be important for receptor stabilization. This dual mode of action by molybdate is supported by observations that 9 S receptor concentration was increased, at the expense of the 4 S binding unit, when molybdate was added to phosphate-buffered cytosols in which the activity of the Ca^{2+}-dependent protease would already have been restricted (T. R., A. M. S., R. H., unpublished). The presence in rat liver of 2 different receptor modifying enzymes, one inhibited by leupeptin or antipain and the other by phosphoramidon or molybdate has been reported [10].

Acknowledgement

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