

Effects of amiloride on the induction of DNA synthesis and casein gene expression in rabbit mammary explants

P Martel¹, LM Houdebine^{2*}

¹ INRA, Laboratoire des Sciences de la Consommation;

² INRA, Unité de Différenciation Cellulaire, 78350 Jouy-en-Josas, France

(Received 23 May 1989; accepted 25 October 1989)

Summary — Amiloride, an inhibitor of Na⁺/H⁺ exchange, was added at various concentrations to the culture medium of rabbit mammary explants. In the concentration range of 100–250 μM, amiloride progressively inhibited ¹⁴C-thymidine incorporation induced by insulin, EGF or prolactin. Up to 250 μM, amiloride, which did not inhibit basal protein synthesis, was not cytotoxic, but it reduced basal DNA synthesis at the highest concentration. Addition of amiloride to the culture medium of mammary explants also strongly inhibited the induction of casein synthesis and casein mRNA accumulation by prolactin. The inhibition by amiloride is therefore not specific of the mitogenic action of prolactin since this drug also prevented its lactogenic action. The data reported here describe a new inhibitory action of amiloride on the transmission of the lactogenic signals.

amiloride / DNA synthesis / casein gene expression

Résumé — Effets de l'amiloride sur l'induction de la synthèse de l'ADN et sur l'expression des gènes des caséines dans des explants mammaires de lapin. L'amiloride, un inhibiteur des échanges Na⁺/H⁺, a été ajouté à des concentrations variables au milieu de culture d'explants mammaires de lapin. Dans l'intervalle de concentration 100–250 μM, l'amiloride inhibe progressivement l'incorporation induite par l'insuline de ¹⁴C-thymidine dans l'ADN, l'EGF et la prolactine. Jusqu'à 250 μM, l'amiloride, qui n'inhibe pas la synthèse protéique basale, n'est toujours pas toxique, mais il réduit la synthèse basale d'ADN aux concentrations les plus élevées. L'addition d'amiloride au milieu de culture des explants inhibe également de manière intense l'induction par la prolactine de la synthèse de caséine et l'accumulation des ARNm correspondants. L'inhibition par l'amiloride n'est donc pas spécifique de l'action mitogène de la prolactine puisque ce composé s'oppose également à son action lactogène. Les données rapportées ici décrivent un nouvel effet de l'amiloride sur la transmission du signal lactogène.

amiloride / synthèse d'ADN / expression des gènes des caséines

* Correspondence and reprints

INTRODUCTION

The major part of the development of the mammary gland takes place during pregnancy. It involves the multiplication and differentiation of the mammary epithelium which leads to the formation of the alveoli, specialized in the synthesis and secretion of milk components. The factors which control the mammary gland growth are multiple and include at least insulin, EGF and prolactin (Martel and Houdebine, 1982). The mitogenic actions of these 3 polypeptides can be studied *in vitro* using rabbit mammary explants (Martel and Houdebine, 1982). In this system, although insulin and EGF are mitogenic when added alone to the synthetic culture medium, the mitogenic action of prolactin is observed only in the presence of insulin and cortisol. In addition, EGF exerts a synergic effect on the mitogenic actions of insulin and prolactin. The 3 polypeptides thus appear to control the multiplication of mammary cells by complementary mechanisms. On the other hand, prolactin not only favours the growth of the mammary gland, but also plays a key role in its differentiation. Indeed, prolactin is essential for the initiation of milk synthesis in all species. Whether both mitogenic and lactogenic actions of prolactin are delivered by the same mechanism is still unknown. The nature of the mediators responsible for the mitogenic actions of insulin, EGF and prolactin still remains to be discovered. One attractive hypothesis is that the mitogenic signal is mediated by intracellular pH changes resulting from ion fluxes. Indeed, ion fluxes are one of the early biochemical events which can be detected just after the addition of growth factors to the culture medium of quiescent cells (Rozengurt, 1983): for example, prolactin, EGF and insulin enhance the influx of the Na⁺ ions in their target cells (Bisbee, 1981; Pouyssegur *et al*, 1982; Moolenaar *et al*, 1984).

Thus, the Na⁺/H⁺ exchange leading to the rise of the intracellular pH has been postulated to play an essential role in the triggering of mitosis by growth factors (Pouyssegur *et al*, 1982; Schuldiner and Rozengurt, 1982). This hypothesis is supported by experiments in which direct measurements of intracellular pH have been carried out. It also relies on the use of amiloride, a strong diuretic drug, which inhibits the Na⁺/H⁺ exchange (Koch and Leffert, 1979; La Belle and Eaton, 1983).

Whether the proposed mechanism is involved in the mitogenic action of one of the 3 polypeptides was tested in rabbit mammary explants. For that purpose, the effect of amiloride on the stimulation of DNA synthesis by insulin, EGF and prolactin was studied. The effect of the drug on the induction of casein synthesis was also evaluated.

MATERIALS AND METHODS

Culture of mammary explants

Mammary fragments were explanted from pregnant rabbits 14 days after mating and cultured for 1 day in medium 199 in the presence of various concentrations of amiloride (Merck Sharp and Dohme-Chibret) and with or without hormones. Amiloride was dissolved in dimethylsulfoxide. Bovine insulin (Sigma), EGF (collaborative Research Incorporation), ovine prolactin (NIH-PS9) and cortisol (Roussel-Uclaf) were added to the culture medium at concentrations of 5 µg/ml, 100 ng/ml, 100 ng/ml and 500 ng/ml, respectively.

DNA synthesis

At the end of the culture, [¹⁴C]thymidine (54 mCi/mmol, CEA) was added to the medium (1 µCi/ml) and the culture was pursued for 2 additional h. The explants were then collected, weighed and digested overnight by 0.5 M NaOH. DNA was precipitated by 5% trichloroa-

cetic acid, collected on glass fiber filters and the radioactivity was estimated by scintillation counting.

Total protein synthesis

At the end of the culture, ^{14}C -amino acids (Chlorella hydrolysate, 1.5–2 mCi/mg, CEA) were added to the medium and the culture was pursued for 2 additional hours. Radioactivity was estimated as described above for DNA synthesis.

Casein synthesis and casein mRNA concentration

At the end of culture, tissue was collected and homogenized in a phosphate buffer containing Triton and the content of β -casein in the homogenate was evaluated using a radioimmunoassay with goat anti-rabbit β -casein and rabbit anti-goat immunoglobulin.

The concentration of β -casein mRNA in total nucleic acids from explants was estimated at the end of cultures by hybridization with a labelled β -casein cDNA probe inserted into a plasmid (Martel *et al*, 1983a).

Quantification of prolactin receptors

^{125}I -hGH taken as the lactogenic hormone (50 $\mu\text{Ci}/\mu\text{g}$) was incubated with crude microsomes prepared after culture of explants in the presence of insulin, with or without prolactin and amiloride. After an overnight incubation at 25 °C (100 000 cpm labelled hormone, with 200 μg membrane protein), membranes were pelleted and the radioactivity specifically bound to membranes was evaluated. When no culture was carried out, crude mammary microsomes were prepared from a lactating rabbit, and incubated with labelled hGH in the presence or in the absence of amiloride as mentioned above.

RESULTS

Effect of amiloride on DNA synthesis

Insulin, EGF and prolactin significantly stimulated [^{14}C] thymidine incorporation

into DNA in rabbit mammary explants. Amiloride was added at various concentrations to the culture medium to study its effects upon the transmission of the 3 mitogenic signals. Amiloride, in the concentration range of 100–250 μM progressively inhibited [^{14}C]thymidine incorporation induced by insulin, EGF or prolactin (fig 1). At these concentrations, amiloride was not cytotoxic since at levels up to 250 μM , basal protein synthesis was not affected (not shown). Nevertheless, amiloride action was not entirely specific since it strongly reduced the basal DNA synthesis, independently of the hormonal induction at the highest concentration. This effect on basal DNA synthesis does not seem to be due simply to the inhibition of the thymidine uptake by explants which re-

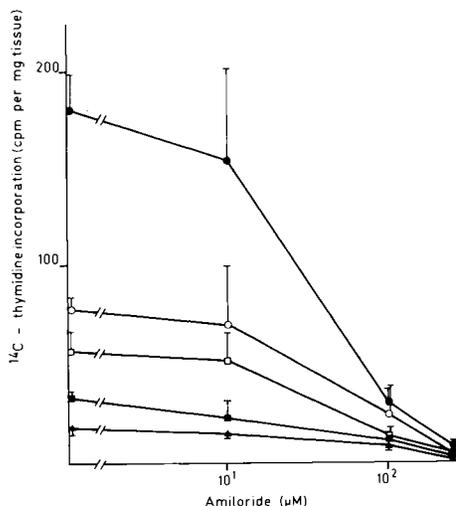


Fig 1. Effect of various concentrations of amiloride on DNA synthesis. Mammary explants were cultured without or in the presence of hormones, alone or in combination. Insulin, EGF, prolactin and cortisol were added at 5 $\mu\text{g}/\text{ml}$, 100 ng/ml, 100 ng/ml and 500 ng/ml respectively. Results, which are the mean (\pm SEM) of 3 independent cultures are expressed as cpm/mg tissue. (\blacktriangle — \blacktriangle) without hormone; (\blacksquare — \blacksquare) EGF; (\square — \square) (insulin); (\circ — \circ) insulin + cortisol; (\bullet — \bullet) insulin + prolactin + cortisol.

mained unaffected by the drug (data not shown). At 100 μM , amiloride did not significantly reduce the basal DNA synthesis whereas it completely inhibited the actions of the 3 mitogenic factors.

Effect of amiloride on casein synthesis

The induction of casein synthesis and the accumulation of the corresponding mRNAs is under the control of prolactin. As a control, it seemed of interest to examine the possible side-effects of amiloride on the transmission of the lactogenic prolactin message to casein genes. Amiloride inhibited the lactogenic action of prolactin as efficiently as its mitogenic action, as judged by its capacity to block the induction of casein synthesis (fig 2). This result was confirmed by the observation that it also prevented the β -casein mRNA accumulation induced by prolactin (fig 3).

Effect of amiloride on prolactin receptors

Since amiloride inhibits both prolactin actions, it is conceivable that its inhibitory effect took place directly at the plasma membrane level. Although amiloride did not affect the binding of prolactin to its microsomal specific receptors (not shown), it slightly reduced the number of prolactin receptors in cultured mammary explants (table I). This slight reduction of the prolactin receptor number could be responsible for a small part of the inhibition by amiloride. Moreover, amiloride did not prevent the down-regulation of the receptors by prolactin (table I).

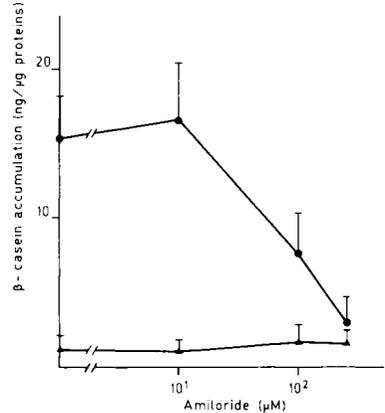


Fig 2. Effect of various concentrations of amiloride on β -casein accumulation. Mammary explants were cultured with insulin (5 $\mu\text{g}/\text{ml}$) and in the presence or the absence of prolactin (100 ng/ml). Results, which are the mean (\pm SEM) of 3 independent cultures are expressed as $\text{ng } \beta$ -casein/ μg protein. (Δ — Δ) insulin; (\bullet — \bullet) insulin + prolactin.

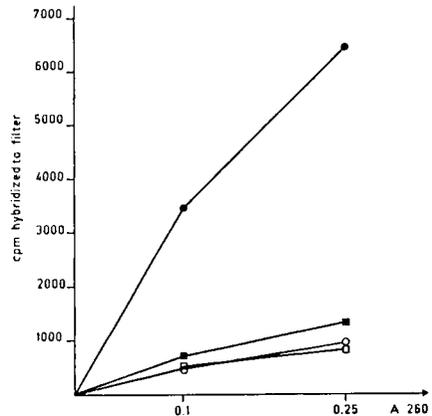


Fig 3. Effect of amiloride on the β -casein mRNA accumulation. Mammary explants were cultured in the presence of insulin 5 $\mu\text{g}/\text{ml}$ and with or without prolactin 100 ng/ml and amiloride 250 μM . After 1 day culture, the β -casein mRNA concentrations were evaluated with a plasmid probe. Results are expressed as cpm of the probe specifically hybridized to the nitrocellulose filters. (O—O) insulin; (□—□) insulin + amiloride; (●—●) insulin + prolactin; (■—■) insulin + prolactin + amiloride.

Table I. Effect of amiloride on the number of prolactin receptors. Mammary explants were cultured for 1 day with or without insulin (I) 5 µg/ml, prolactin (PRL) 100 ng/ml and amiloride 250 µM. The number of prolactin receptors was evaluated by measuring the capacity of the membranes, prepared at the end of the culture, to bind prolactin. Results are expressed as the percentage of the hormone specifically bound to membranes.

<i>Culture medium</i>	<i>Prolactin receptors (% specific binding)</i>
Control	12.4
I	17.2
I + PRL	8.6
I + amiloride 250 µM	13.7
I + PRL + amiloride 250 µM	7.4

DISCUSSION

The data reported here clearly indicate that amiloride inhibits the mitogenic action of prolactin. This observation is compatible with the hypothesis that Na⁺/H⁺ exchanges are involved in the mitogenic action of prolactin as seems to be the case for other mitogenic factors (Pouyssegur *et al*, 1982; La Belle and Eaton, 1983). Surprisingly, however, amiloride also blocks the lactogenic action of prolactin. This either means that Na⁺/H⁺ exchange is involved in the transmission of the lactogenic signal to genes or that amiloride interferes at an early unknown step of the prolactin mechanism of action which is common to the mitogenic and lactogenic signals of the hormone. If this is the case, the effects of amiloride depicted in the present report are possibly of a new type and may be added to others indirectly related to Na⁺/H⁺ exchange (Fehlmann *et al*, 1981; Lubin *et al*, 1982; Holland *et al*, 1983). Interestingly, somewhat similar conclusions have been drawn from other experiments which demonstrated that colchicine (Martel and Houdebine, 1982), phorbol esters (Martel *et al*, 1983b) and sodium butyrate (Martel *et al*, 1983a) inhibited prolactin actions.

Colchicine and related drugs, phorbol esters, sodium butyrate and amiloride have no clear similarity in their chemical structure and it is not easy to imagine how these drugs could interfere with the prolactin mechanism of action. All these compounds are potentially interesting tools to study in more detail the action of prolactin at the molecular level.

ACKNOWLEDGMENTS

The authors wish to thank Mrs Marie-Louise Fontaine, Valérie François and Claudine Puisant for their technical help. This work was supported by the financial help of CNRS and INSERM and the Biotechnology Action Programme of the European Community.

REFERENCES

- Bisbee CA (1981) Prolactin effects on ion transport across cultured mouse mammary epithelium. *Am J Physiol* 9, 110-115
- Fehlmann M, Samson M, Koch KS, Leffert HL, Freychet P (1981) Amiloride inhibits protein synthesis in isolated rat hepatocytes. *Life Sci* 28, 1295-1302

- Holland R, Woodgett JR, Grahame Hardie D (1983) Evidence that amiloride antagonizes insulin-stimulated protein phosphorylation by inhibiting protein kinase activity. *Febs Lett* 154, 269-273
- Koch KS, Leffert HL (1979) Increased sodium ion influx is necessary to initiate rat hepatocyte proliferation. *Cell* 18, 153-163
- La Belle EF, Eaton DC (1983) Amiloride-inhibited Na^+ uptake into toad bladder microsomes is Na^+ H^+ exchange. *Biochim Biophys Acta* 733, 194-197
- Lubin M, Cahn F, Coutermarsh BA (1982) Amiloride, protein synthesis and activation of quiescent cells. *J Cell Physiol* 113, 247-251
- Martel P, Houdebine LM (1982) Effect of various drugs affecting cytoskeleton and plasma membranes on the induction of DNA synthesis by insulin, epidermal growth factor and prolactin in mammary explants. *Biol Cell* 44, 111-116
- Martel P, Houdebine LM, Teyssot B (1983) Effect of sodium butyrate on the stimulation of casein gene expression by prolactin. *FEBS Lett* 154, 55-59
- Martel P, Houdebine LM, Teyssot B, Djiane J (1983) Effects of phorbol esters on multiplication and differentiation of mammary cell. *Biol Cell* 49, 119-126
- Moolenaar WH, Yarden Y, De Laat SW, Schlesinger J (1982) Epidermal growth factor induces electrically silent Na^+ influx in human fibroblasts. *J Biol Chem* 257, 8502-8506
- Pouyssegur J, Chambard JC, Franchi A, Paris S, Van Obberghen-Schilling E (1982) Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: coupling to ribosomal protein S6 phosphorylation. *Proc Natl Acad Sci USA* 79, 3935-3939
- Rozenfurt E (1983) Signalling mitogenesis in 3T3 cells: role of monovalent ion fluxes and cyclic nucleotides. *Cell Biol Reprod* 7, 495-496
- Schuldiner S, Rozenfurt E (1982) Na^+/H^+ antiport in Swiss 3T3 cells: mitogenic stimulation leads to cytoplasmic alkalinization. *Proc Natl Acad Sci USA* 79, 7778-7782