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Functional domains essential for Gs activity in prostaglandin EP2 and EP3 receptors

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Abstract

The interaction of cell surface hormone receptors with heterotrimeric G proteins is crucial for hormonal actions. The domains of the receptor, which interact with and activate G protein, have been extensively studied. However, precise molecular mechanisms underlying regulation of the receptor-induced G protein activation are still poorly understood. Prostaglandin E_2 (PGE_2) receptors comprise of four subtypes, EP1, EP2, EP3 and EP4. Among them, EP2 and EP4 couple to Gs and EP3 to Gi. To assess the functional domains essential for Gs activation in prostanoid receptors, EP2, EP3β and each intracellular loop- (IC-) interchanged EP2/EP3 chimeras were tested for agonist binding and functional responses. In EP2 receptor, substitution of IC1 or IC3 resulted in loss of binding activity, while substitution of IC2, N- (IC2N) or C-terminal half region of IC2 (IC2C) had no effects on the binding activity. Wild-type EP2 and IC2C-substituted EP2 showed agonist-induced Gs activity, but IC2- and IC2N-substituted EP2 failed to elicit Gs activity upon agonist stimulation. On the other hand, in EP3 receptor substitution of IC1 resulted in loss of PGE_2 binding, while substitution of IC2, IC3, IC2N or IC2C had no effects on binding activity. Wild-type EP3β, IC3- or IC2C-substituted EP3 failed to show Gs activity upon agonist stimulation, but IC2- or IC2N-substituted EP3 chimera showed agonist-dependent Gs activity. These results indicated that the second intracellular loop of the EP2 plays an essential role in activation of Gs.

Keywords: G protein; PGE_2; Prostaglandin E_2 subtypes

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Introduction

The interaction of cell surface hormone receptors with heterotrimeric G proteins is crucial for hormonal actions (Gilman, 1987). Most G protein-coupled rhodopsin-type receptors share conserved structural features, consisting of seven hyprophobic domains and three intracellular loops and one carboxyl-terminal tail (Dohlman et al., 1991). The domains of the receptors which interact with and activate G proteins have been extensively studied, and specific regions in the second and third intracellular loops were shown to function in G protein coupling (Conklin and Bourne, 1993; Wess, 1998; Pierce et al., 2002). However, precise molecular mechanisms underlying regulation of the receptor-induced G protein activation are still poorly understood.

Prostaglandin E_2 (PGE_2), the most well-known prostanoid, exhibits a broad range of biological actions in diverse tissues through their binding to specific receptors on plasma membrane (Negishi et al., 1995a). We and other groups have revealed the primary structures of eight types of prostanoid receptors including four subtypes of PGE receptors (EP1, EP2, EP3 and EP4) and demonstrated that they belong to the G protein-coupled rhodopsin-type receptor superfamily (Negishi et al., 1995b). Although four EP subtypes are different in G protein coupling, they have several unique features specific to prostanoid receptors, in addition to those in common with other rhodopsin-type receptors; for example, they contain quite less number of basic or acidic amino acids throughout the putative transmembrane domains (Narumiya et al., 1999). To assess the roles of such unique structural features, we have investigated the properties of receptors with mutations within such unique regions and demonstrated that the Arg residue within the putative seventh transmembrane domain conserved in all prostanoid receptors is important not only for interaction with the carboxylic acid group of agonists, but also for particular signal activation (Chang et al., 1997a and 1997b; Hizaki et al., 1997; Ichikawa et al., 1997). Furthermore, we have found that Asp residue within seventh transmembrane domain of EP3 receptor plays a key role for governing G protein association and activation (Satoh et al., 1999). Thus, the structurally-close members of G protein-coupled receptor subfamily like prostanoid receptors are useful materials for understanding the structure and function relationship including G protein activation.

In order to examine structural requirements for signal transduction, we constructed a series of EP2/EP3 chimeric receptors and examined loss of Gs activity in EP2 and gain of Gs activity in EP3.

Materials and methods

Construction of the mEP2, mEP3β, EP2-based, and EP3-based hybrid receptors

The functional cDNAs for mouse EP2 (mEP2), and EP3β (mEP3β) were previously cloned in our laboratory (Katsuyama et al., 1995; Sugimoto et al., 1993). The construction of pcDNA3-based expression plasmids (Invitrogen, Carlsbad, CA) encoding for wild-type mEP2 and mEP3β has been described previously (Hatae et al., 2002). Various kinds of EP2/EP3 chimeric receptors were prepared by standard PCR-based mutagenesis techniques (QuickChange Site-directed mutagenesis kit, Stratagene, La Jolla, CA). In EP2-based chimeras, the following mEP2 receptor sequences were replaced with the corresponding mEP3β receptor segments; EP2-IC1, mEP2 47–67 → mEP3β 50–64; EP2-IC2, mEP2 136–151 → mEP3β 133–148; EP2-IC3, mEP2 222–262 → mEP3β 231–

256; EP2-IC2N, mEP2 136–143 → mEP3β 133–140; EP2-IC2C, mEP2 144–151 → mEP3β 141–148. In EP3-based chimeras, the following mEP3β receptor sequences were replaced with the corresponding mEP2 receptor segments; EP3-IC1, mEP3β 50–64 → mEP2 47–67; EP3-IC2, mEP3β 133–148 → mEP2 136–151; EP3-IC3, mEP3β 231–256 → mEP2 222–262; EP3-IC2N, mEP3β 133–140 → mEP2 136–143; EP3-IC2C, mEP3β 141–148 → mEP2 144–151. For transfection using the LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA), HEK293 cells were incubated at 37 °C for 4 h with a transfection mixture containing 5 μg of DNA and 15 μl of lipofectAMINE 2000 reagent.

PGE_2-binding assay and measurement of cAMP formation in HEK293 cells

Sulprostone and butaprost were generous gift from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. The harvested HEK293 cells expressing each receptor were homogenized using a Potter-Elvehjem homogenizer. After centrifugation at 250,000 × g for 20 min, the pellet suspension was used for the [^{3}H]PGE_2-binding assay. The membrane (50 μg) was incubated with various concentrations of [^{3}H]PGE_2 at 30 °C for 1 h, and [^{3}H]PGE_2 binding was determined using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. Cyclic AMP levels in HEK293 cells were determined as reported previously (Sugimoto et al., 1993). The receptor-expressing HEK293 cells cultured in 24-well plates (5×10^5 cells/well) were washed with saline and preincubated for 10 min. Reactions were started by the addition of test reagents along with 100 μM Ro-20-1724. After incubation for 10 min at 37 °C, reactions were terminated by the addition of 10% trichloroacetic acid. The content of cAMP in the cells was measured by radioimmunoassay with cAMP assay system (Amersham Biosciences Corp., Piscataway, NJ).

Results and Discussion

As a first step to elucidate what domains of putative intracellular regions contribute to Gs coupling and activation in prostanoid receptors, we generated EP2/EP3 chimeric receptors and examined each receptor-elicited cAMP producing activity. EP2 and EP3 receptors are coupled to stimulation and inhibition of adenylate cyclase, respectively. In mouse EP3, there are three isoforms with different C-terminal domains, EP3α EP3β and EP3γ (Ichikawa et al., 1997). These isoforms are predominantly coupled to Gi, but they are different in their agonist-induced Gs activity. Among them, we used mouse EP3β (mEP3β) which shows fully agonist-dependent Gi activity without Gs activity.

By focusing on the roles of intracellular loop 1 to 3, we generated EP2-based (EP2-IC1, EP2-IC2 and EP2-IC3) and EP3-based (EP3-IC1, EP3-IC2 and EP3-IC3) chimeric receptors expressed in HEK293 cells (Fig. 1A). For example, EP2-IC1 means EP2 receptor with its intracellular loop 1 replaced with corresponding region of EP3β. We first examined whether each hybrid receptor showed appropriate binding activity for PGE_2 by using crude membranes of HEK293 cells (Table 1). In EP2-based chimeras, EP2-IC2 showed high level of specific PGE_2 binding, and Scatchard analysis yielded a dissociation constant (Kd) close to that of wild-type EP2 receptor, but EP2-IC1 and EP2-IC3 showed no specific PGE_2 binding activity. In EP3-based chimeras, EP3-IC2 and EP3-IC3 showed Kd values comparable to that in EP3β, but EP3-IC1 showed no significant level of specific binding. Since the

binding affinities for PGE_2 both in EP2 and EP3 were unaffected by the interchange of intracellular loop 2, this region might be flexible for receptor molecules to take conformation necessary for high affinity binding activity. In contrast, the binding activities for PGE_2 in both EP2 and EP3 were lost by the interchange of intracellular loop 1, this region may be critical for EP-specific formation of PGE_2 binding pocket. The difference in the effects of replacement of intracellular loop 3 may reflect rigidity of this region in EP2 and/or flexibility of the region in EP3. When we performed cAMP production assay,

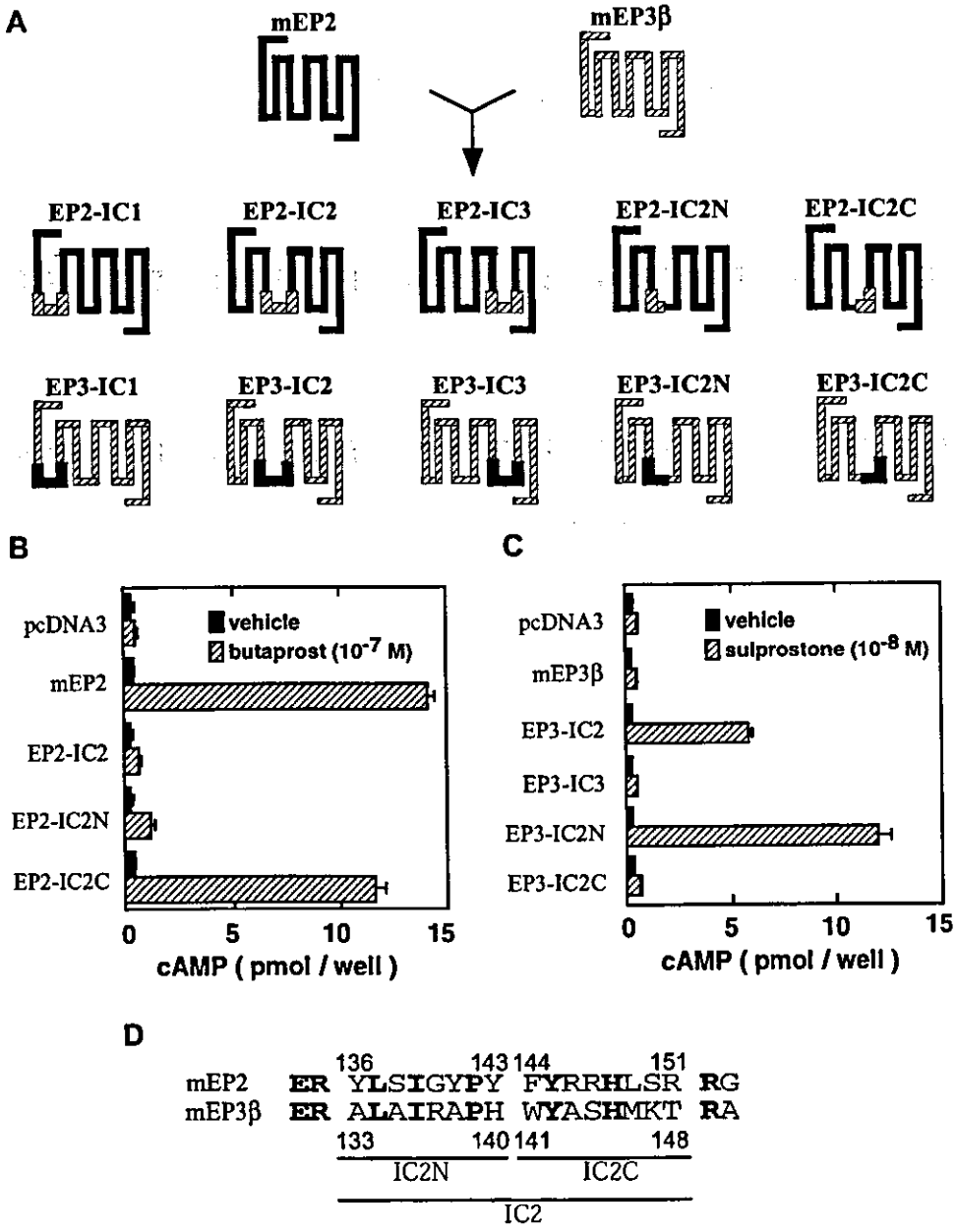

Table 1
Summary of binding properties in mEP2, mEP3β and their mutant receptors

Receptor	Kd (nM)	Bmax (pmol/mg protein)
mEP2	19	0.93
EP2-IC1	n.d.	n.d.
EP2-IC2	23	1.1
EP2-IC3	n.d.	n.d.
EP2-IC2N	16	1.0
EP2-IC2C	12	0.80
mEP3β	2.3	2.7
EP3-IC1	n.d.	n.d.
EP3-IC2	1.5	2.5
EP3-IC3	1.4	0.90
EP3-IC2N	2.9	3.6
EP3-IC2C	2.0	1.9

n.d., not detected.

wild-type mEP2 elicited Gs activity, while EP2-IC2 failed to elicit this activity upon butaprost treatment (Fig. 1B). The results suggested that intracellular loop 2 of the EP2 might play a role in Gs activation. These results also raised the possibility that the EP3-IC2 having intracellular loop 2 of EP2 might gain Gs activity. To explore this possibility, we performed cAMP formation assay in EP3-IC2-expressing cells (Fig. 1C). EP3β did not elicit Gs activity upon sulprostone treatment, while EP3-IC2 showed significant level of cAMP accumulation. In order to narrow the key regions for Gs activation, we further constructed EP2 and EP3 receptors with their N- or C-terminal half of intracellular loop 2 replaced with the corresponding region of the other receptor (EP2-IC2N, EP2-IC2C, EP3-IC2N and EP3-IC2C). As expected, these hybrid receptors showed binding affinities similar to those of wild-type receptors (Table 1). In EP2-IC2C-expressing cells, significant amount of cAMP formation was observed upon butaprost stimulation as in wild-type EP2-expressing cells (Fig. 1B). However, EP2-IC2N failed to show Gs activity. In contrast, EP3-IC2C did not show agonist-induced cAMP formation, while EP3-IC2N elicited Gs activity in response to sulprostone (Fig. 1C). These results suggested that the N-terminal half of the intracellular loop 2 (IC2N) of the EP2 receptor (eight amino acid sequence in Fig. 1D) is critical for elicitation of Gs activity. Alternatively, the IC2N of the EP3 may inhibit Gs activity. The IC2N comprises of 8 amino acids, and 3 amino acids were identical between mEP2 and mEP3β. In addition,

Fig. 1. Structures, Kd values and agonist-dependent cAMP producing activities of mutant receptors. A, diagrams showing structures of mEP2, mEP3β, and 10 mutant receptors used in this study. The part of the receptors derived from mEP2 is shown by a closed box, and that from mEP3β is shown by a open box. The binding affinities for [^{3}H]PGE_2 in mEP2, mEP3β and mutant receptors expressed in HEK293 cells were assessed by Scatchard plot analysis, and the Kd values were shown below diagrams. B and C, agonist-dependent cAMP formation in HEK293 cells expressing mEP2 and EP2-based mutant receptors (B) and in HEK 293 cells expressing mEP3β and EP3-based mutant receptors (C). The HEK293 cells expressing each receptor or pcDNA3-transfected HEK293 cells were seeded at 8×10^5 cells / well and cultured for 24 h until assay. For mEP2 and EP2-based mutant receptors, the cells were stimulated for 10 min by adding media with or without 0.1 μM butaprost, an EP2-selective agonist (B). For mEP3β and EP3-based mutant receptors, the cells were stimulated for 10 min by adding media with or without 0.01 μM sulprostone, an EP3-selective agonist (C). Intracellular cAMP levels were measured by radioimmunoaasay. D. Amino acid sequences in the second intracellular loop 2 of the mEP2 and mEP3β. The identical amino acids in the two receptors are presented by bold letters. The cAMP contents were determined as described in Materials and methods. The results shown are the means ± S.E.M. for triplicate determinations.

rat EP2 receptor contains Ala residue at position 138 instead of Ser, indicating that Ser-138 is less important for Gs activation. Therefore, the key amino acid(s) critical for Gs activity is likely to be either of Tyr-136, Gly-140, Tyr-141 and Tyr-143. We are now examining the effects of point mutation at each one of these four amino acids on Gs activity of EP2.

The previous reports have provided strong evidence that the intracellular loop 2 represents one of the key regions regulating the selectivity of receptor/G protein interactions (Wess, 1998). In the current study, replacement of the IC2N region of the EP3 with the corresponding EP2 sequence yielded a mutant receptor that gained productive coupling to Gs. Among the four EP2-specific amino acids in this region, Tyr-143 is one of the most likely candidates. This is because Moro et al. (1993) proposed that hydrophobic amino acid at this position plays a key role in receptor-G protein coupling. However, they proposed this amino acid might serve as a general site relevant to G protein coupling, whereas a selectivity of G-protein coupling being governed by other domains. Further analysis is required to clarify the roles of IC2N in Gs coupling to EP2 and EP3 receptors.

Conclusion

The N-terminal half region of the intracellular loop 2 is required for Gs coupling in EP2 receptor and substitution with this domain alone can be sufficient to confer Gs-coupling property on EP3 receptors.

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MOUSE MAMMARY EPITHELIAL HISTAMINE SYSTEM

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Histamine is suggested to play a role in mammary gland growth regulation, differentiation and functioning during pregnancy and lactation. Two pools of histamine are thought to be involved in these processes: mastocyte- and epithelial cell related histamine. In the present study we focused on epithelial cells. Immunohistochemistry has shown that the epithelial cells positive for histamine and L-histidine decarboxylase (HDC), the primary enzyme regulating histamine biosynthesis, were mainly found in cells forming alveolar structures in the mammary gland. Cultured primary mouse mammary epithelial cells (MMEC) expressed strong HDC immunoreactivity, especially dividing cells and non-differentiated ones. Histidine decarboxylase activity undergoes significant changes during pregnancy and lactation. Pregnancy associated intensive growth of the mammary gland coincided with an increase and the first days of lactation with a decrease of HDC protein expression. Binding studies with mammary tissue membranes and epithelial cell membranes revealed the presence of H_1 and H_3 but not H_2 receptors. Summarizing, our data have shown that mammary epithelial cells are capable of synthesizing and excreting histamine and they bear histamine receptors. These findings further substantiate the role of histamine in mammary gland physiology.

Key words: *mouse mammary gland, epithelial cells, L- histidine decarboxylase, histamine, histamine receptors*

INTRODUCTION

The original observation of a high level of histamine in mouse mammary glands (1), suggested a possible role for this amine in mammary gland function and development. Histamine is known to exert various effects in physiological

and pathological reactions such as: smooth muscle contraction, gastric acid secretion, cell growth, neurotransmission and inflammation (2). Histamine is an iminoamine synthesized locally from L-histidine by a specific enzyme L-histidine decarboxylase (HDC, EC 41.1.22). The histamine is inactivated by two main enzymes: histamine N-methyltransferase (HMT, EC 2.1.1.8) via methylation (brain and periphery) and diamine oxidase (DAO, EC 1.4.3.6) via oxidative deamination (peripheral tissues only) (3).

The actions of histamine are mediated through the activation of membrane-associated G protein coupled receptors: H_1, H_2, H_3 and H_4. Histamine receptors in mice have been characterised pharmacologically and murine H_1 and H_2 receptors have been cloned (4, 5). In addition to well known histamine containing cells, i.e. mast cells, enterochromaffin-like cells and neurons, studies have demonstrated the presence of histamine systems in some other tissues and cells, such as blood vessel endothelial cells (6, 7) or mammary gland epithelial cells (8-10). The latter observation and the effects of HDC inhibitors and H_2 receptor antagonists on a growth of mammary cancer cells *in vitro*, has led to the development of clinical research in this area (11, 12).

In the first study on the metabolism of histamine in mammary glands (13), it was demonstrated that the mammary histamine system changes markedly during the estrous cycle as well as during pregnancy and lactation. Potent physiological effects of histamine on mammary glands were shown in vivo and *in vitro* (14). Histamine receptors agonists on their own or in the presence of oxytocin increased milk secretion. The H_3-receptor antagonist - FUB 181 had an additive effect on oxytocin stimulated milk secretion from goat mammary glands (15). Although the main histamine immuno-reactive sites in mammae were mast cells, some histamine-positive signals were also found in glandular epithelium and in the stroma (16).

In this paper we present the results of a study of the histamine system and HDC protein expression profile in mouse mammary glands at various physiological states.

MATERIALS AND METHODS

Animals

Virgin female mice (Mus musculus) (BALB/C, 20 g body wt) were used. Animal procedures complied with the Polish legislation concerning experiments on animals and were approved by the local care committee.

Pregnancy and lactation

Female mice were housed with males of proven fertility for three days accompanied by daily microscopic examination of vaginal smears. The presence of spermatozoa and/ or vaginal plug was regarded as the first day of pregnancy. Pregnancy day was further verified at autopsy by embryo development stage. The parturition day was designated as the first lactation day.

Cell isolation procedure

Mammary epithelial cells (MMEC) were obtained from resting animals as well as pregnant and lactating animals and were isolated from the abdominal mammary glands. In brief, dissected tissues were digested with collagenase (collagenase IV 2 mg/ml, hyaluronidase 100U/ml in DMEM supplemented with antibiotic-antimycotic solution (all reagents from Sigma, St. Louis, MO, USA) for 2 h at 37°C. Finally, the cells were purified using differential sieving and repetitive DMEM washing and sedimentation.

Primary cell culture

Isolated MMEC were cultured in DME/F12 medium supplemented with 10% horse serum, hydrocortisone (0.5 µg/ml), insulin (10 µg/ml) and antibiotic-antimycotic solution. They were incubated at 37°C in 3% CO_2 humidified environment.

For histamine synthesis assays, cells were cultured in complete medium based on DMEM instead of DME/F12. HDC activity assays were performed after approximately 4-5 days of culture when cells reached confluence on 16 millimetres (24-well) (Corning, NY, USA) or 60 millimetres in diameter culture dishes (Nunc, Denmark).

For immunocytochemistry, cells were grown for 9 days on 8-well Lab-Tek Chamber Glass Slides (Nunc, Inc., IL, USA). For receptor binding studies cells were incubated up to 72 h on 60 or 120 millimetres in diameter culture dishes (Nunc, Denmark).

Histamine synthesis and secretion assay

Twenty four h after cell plating and every 24 h thereafter during a 72 h experiment, the cells and medium were harvested and centrifuged at 20 g. Postculture medium was removed and the cells were purified by repeated washing in cold PBS and centrifugation. Finally, the cells were resuspended in 0.3 ml of PBS and lysed by three cycles of freezing and thawing. Then, cell homogenates and culture medium were deproteinized by boiling at 100°C for 10 minutes and spun down at 12,000 g for 10 minutes. Histamine levels in supernatant and postculture media were measured by RIA (Immunotech, France) and expressed per 10^6 cells.

Histidine decarboxylase activity assay

The epithelial cell histidine decarboxylase activity was estimated after 4-5 days cell culture (17). Harvested and purified cells (as described above) were suspended in enzyme-extraction buffer (0.1 M potassium buffer, pH 6.8, containing 1mM of dithiothreitol and 0.1 mM pyridoxal 5'-phosphate) and lysed by three cycles of freezing and thawing followed by sonication (5 minutes). The homogenates were centrifuged at 12,000g for 20 minutes and resulting supernatants dialysed. Dialysis was carried out against extraction buffer for 60 minutes at 4°C with a fresh buffer change every 20 minutes. HDC activity was assayed with 25 mM L-histidine. Incubation was for 1 hour at 37°C. The reaction was stopped by heat enzyme inactivation (100°C, 10 minutes). Newly synthesised histamine was used as a measure of the enzyme activity. It was estimated with a radioenzymatic assay employing histamine N-methyltransferase (HMT) (18) and adenosyl-L-methionine S-[methyl - ^{3}H] (New Nuclear, Boston, MA, USA) as a donor of methyl group (19).

Histamine- , histidine decarboxylase - immunohistochemistry

Immunostaining for histamine was carried out on 20 µm thick cryostat sections as described previously (20). Fixed samples were washed in 0.01 M PBS containing 0.125% Triton X-100 (PBS

- T) followed by incubation with a histamine antiserum diluted (1: 2,000) in PBS-T containing 1% normal goat serum overnight at 4°C in humidified chamber. After washing in PBS-T the samples were incubated for 1 hour at room temperature with fluorochrome conjugated goat anti-rabbit IgG (1:500, Alexa 488 GAR 2 mg/ml, Molecular Probes, Eugen, USA). Control samples were incubated with the same serum pre-adsorbed with histamine-BSA conjugate. After the final washes in PBS-T and PBS the samples were mounted in 50% glycerol (in PBS) and analyzed with Leitz Aristoplan fluorescence microscope.

Histidine decarboxylase immunostaining was carried out on 3-4 μm cryostat sections. Sections were fixed in acetone for 10 minutes at room temperature. After pretreatment with 0. 05 M TBS (Tris buffer solution, pH 7.6) slides were incubated with rabbit anti-GST-HDC fusion protein polyclonal antibody (1:500) (21) for 1 hour at room temperature. TBS washed slides were followed either by secondary horse anti-rabbit IgG and visualised with an ABC kit (Novocastra Laboratories Ltd., UK) or secondary fluorescein conjugated swine anti-rabbit IgG (Dako, Denmark). Washed with TBS samples were mounted in mounting mediums and examined with Olympus CX41 microscope or Jena Lumar (Carl Zeiss Jena) fluorescence microscope.

Histidine decarboxylase - immunocytochemistry

Histidine decarboxylase immunostaining was examined using primary mammary epithelial cells culture grown on Lab-Tech chamber glass slides. Cultures were washed briefly in PBS (pH7.4) and fixed in acetone for 10 minutes at room temperature. Immunostaining was carried out as described above with an ABC kit visualisation.

Saturation binding experiments

Saturation binding assays were essentially performed as described previously (22) with some changes. Cell membranes (400 μl; gland: 0.5-1.6 mg protein/ml; cells: 0.4-1.2 mg protein/ml) were incubated for 30 min with continuous shaking (30°C) in triplicate in a total volume of 500 μl with increasing concentrations of the specific histamine receptors H_1, H_2 and H_3 radioligands. For the H_1 receptor assay isotopic ligand [^{3}H]-Mepyramine (0.1-30 nM) (29.0-30.0 Ci/mmol, Amersham Pharmacia Biotech, UK) and "cold" ligand Triprolidine dihydrochloride 2 μM (Sigma) were used. For the H_2 receptor assay ligands were: [^{3}H]-Tiotidine (0.5-30 nM) (89.6 Ci/mmol, New England Nuclear, Boston, MA, USA) and 5 μM Histamine dihydrochloride (Sigma) respectively. [^{3}H]-R(-)-α-Methylhistamine (0.15-9 nM) (29.0-35.0 Ci/mmol, Amersham Pharmacia Biotech, UK) and 1 μM Histamine were applied for the H_3 receptor assay.

Data analysis

The data are presented as mean ± SEM for n experiments. Comparisons among groups were carried out using analysis of variance (ANOVA) and *post hoc* Newman-Keuls test. Saturation data was analysed using the non-linear curve fitting program (GraphPad Prism 3.0).

RESULTS

Epithelial cell HDC activity and histamine concentration changes during pregnancy and lactation

The highest level of HDC activity was 409.29 ± 47.41 pmol/h/mg protein and was measured in epithelial cell cultures derived from 10-12th day of pregnancy

(*Fig.1A*). It was almost 4 fold higher than in epithelial cells from resting mammary glands (109.18 ± 24.48 pmol/h/mg protein). A plateau of HDC activity was seen at between 14th-16th and the 20th day of pregnancy: 146.78 ± 13.74 and 104.63 ± 11.46 pmol/h/mg protein respectively. A marked reduction in enzyme

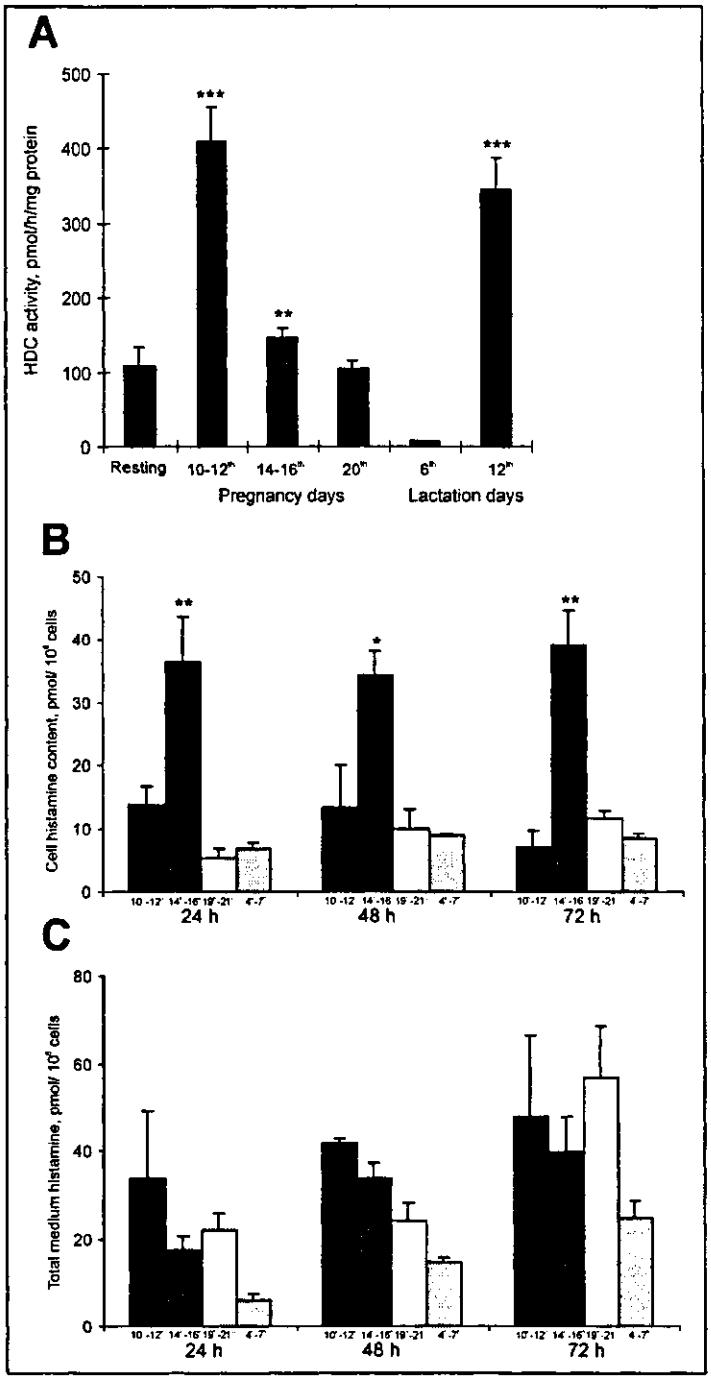

Fig. 1. (A) L- Histidine decarboxylase (HDC) activity in cultured mammary epithelial cells derived from female mice mammary glands of different physiological stages. Each point represents the mean ± SEM of 4 - 6 determinations. *** $p<0.001$ compared with resting, 10-12th, 14-16th, 20th day of pregnancy and 6th day of lactation. ** $p<0.01$ compared with 6th day of lactation. (B) Intracellular and extracellular histamine (C) in cultured mammary epithelial cells. Cultures were prepared from mammary glands of pregnant (10th-12th, 14th-16th, 19th-21st day) and lactating (4th-7th day) mice. Cell and medium contents were measured after 24 hr, 48 hr and 72 hr of incubation. * $p<0.05$ and ** $p<0.01$ compared with time - corresponding counterparts.

activity was found after parturition on the 6th day of lactation (6.71 ± 0.75). On 12th day after parturition the HDC activity again reached a high level of 344.92 ± 43.64 pmol/h/mg protein.

Throughout the 72 hour experiments, cell histamine contents in epithelial cells from mammary gland at 14-16th day of pregnancy were significantly higher (39.21 ± 11.09 pmol/10^6 cells) than in corresponding cell cultures obtained from the gland at the 10-12th, the 19-21st days of pregnancy and the 4-7th day of lactation (7.09 ± 3.55; 11.52 ± 2.37; 8.38 ± 1.41 pmol/10^6 cells, respectively) (*Fig.1B*). The epithelial cell cultures derived from mammary gland at the 10-12th and at the 19-21st days of pregnancy showed higher histamine release from the cells, as judged by medium and cell histamine contents. The cells from the 14-16th day of pregnancy showed balanced histamine synthesis and secretion during the whole experiment. The histamine concentrations in the medium tended to increase in a time-dependent manner in all cell cultures, and the maximum values were recorded after 72 h culture (*Fig.1C*).

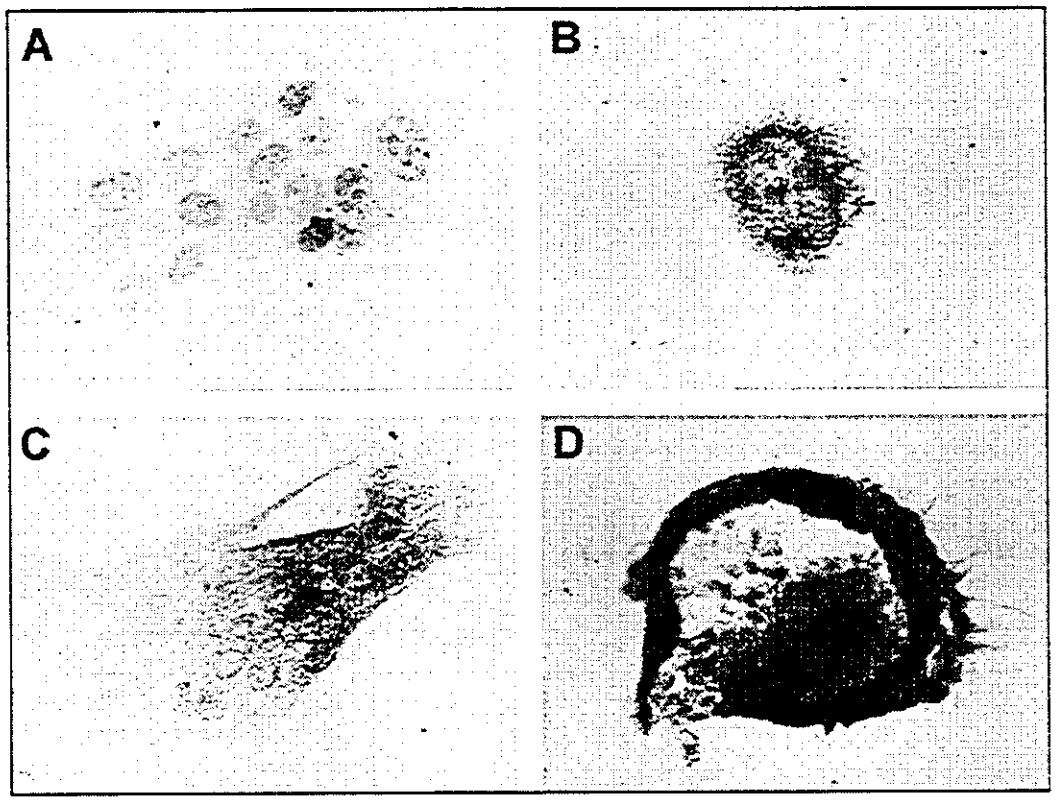

Fig. 2. In situ staining of cultured mammary epithelial cells with anti-mouse-HDC antibody. (A) Negative controls. (B) Single, non-differentiated epithelial cell, positively stained. (C) Small colony of epithelial cells showing different expression of enzyme protein. (D) Staining of organoid. The magnification of the photographs is as follows: A, C-400x; B-800x; D-200x.

HDC immunocytochemistry in primary epithelial cell culture

To visualise and localise HDC in mammary gland epithelial cells, primary epithelial cell cultures were used. Cells isolated from mammary glands from

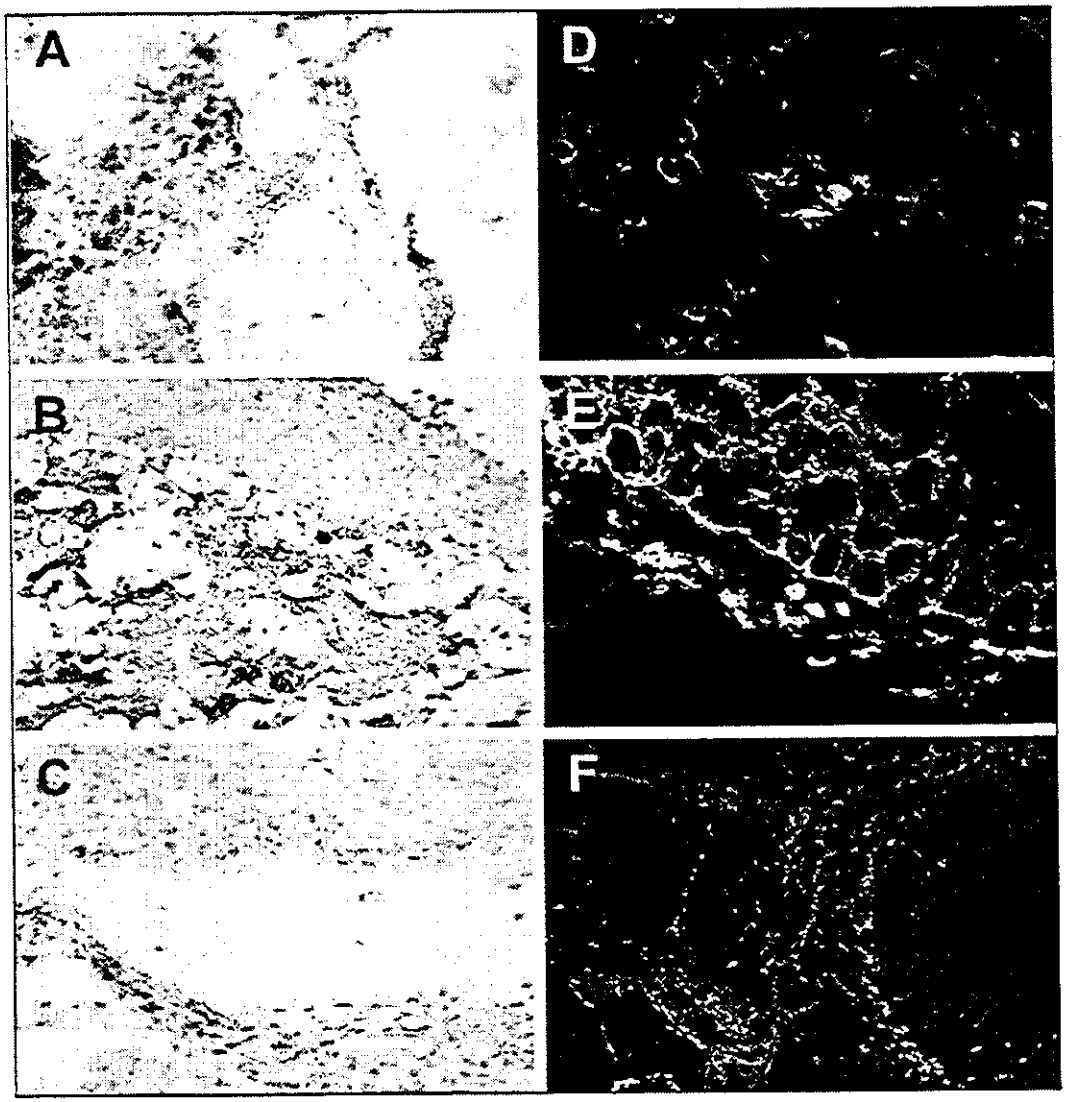

Fig. 3. Left panel shows the results of HDC immunohistochemistry of mouse resting, pregnancy and 5th day of lactation mammary glands. (A) Resting mammary gland, positively stained, single mast cell in the centre. (B) 14-16th day of pregnancy mammary gland sections, positively stained alveolar epithelium is seen. (C) HDC - immunopositive cells spread in the vicinity of the duct, 5th day of lactation. Right panel presents the indirect histamine - immunofluorescent histochemistry of 15th day pregnancy mammary gland. (D) Positive staining of few epithelial cells in alveolar epithelium. (E) Positive staining of alveolar structures (upper part). A few mast cells showing strong signals of immunofluorescence are seen in intralobular stroma. (F) Positively stained alveolus is seen in the lower part of the left side of the photograph. The magnification of the photographs is as follows: A,D-400x; B-200x; C-300x; E-100x; F-40x.

animals in the 14-16th day of pregnancy, were cultured and stained as described in Materials and Methods. Under the conditions employed dividing or single not-differentiated cells were rich in HDC molecules (*Fig.2B, C*). On the other hand, single flattened differentiated cells exhibited low or no HDC protein expression.

Histamine and HDC immunohistochemistry in mouse mammary gland sections

The study on histamine/HDC immuno-localization in mammary gland was focused on glandular tissue. Signals of histamine immunofluorescence were found in alveolar structures of the mammary gland and they were mostly associated with epithelium (*Fig.3D,F*). Stronger histamine-immunoreactivity was confined to mast cells which were numerous in connective tissue and diffused in glandular tissue (*Fig.3E,F*)

HDC-immunopositive cells with a mast cell like granular pattern of staining were clearly visible in preparations visualised with the ABC system. They were found in connective tissue of resting (*Fig.3A*) and lactating mammary glands (data not shown). Epithelial cells of alveolar formations displayed only a weak signal, barely distinguishable from the background (*Fig.3B*). Strong HDC immunoreactivity was also found in the vicinity of the ducts from lactating

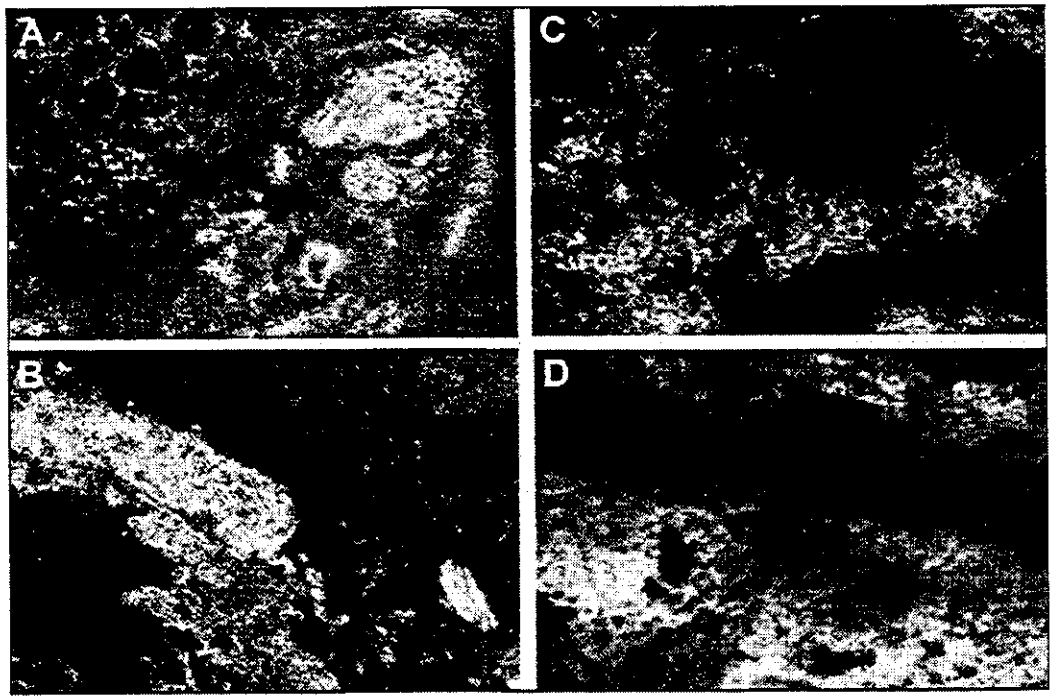

Fig. 4. Stage - course HDC - immunofluorescent histochemistry of mouse mammary gland. (A) 10-12th day of pregnancy, strong immunoreactivity in the alveolar structures. (B) 19th day of pregnancy, diminished immunoreactivity in alveolar epithelium as compared to the 10-12th day of pregnancy. (C) 5th day of lactation, lack of specific immunofluorescence. (D) Immunopositive signals appear again in alveolar epithelium on 10th day of lactation. Magnification: 150x.

mammary glands (*Fig.3C*). When the fluorescence technique was applied, the epithelial immunopositivity was found in cells of alveolar formations and lining duct cells in pregnancy and lactating mammary glands (*Fig.4*).

Histamine receptors in mammary gland tissue and primary epithelial cell culture

We used the H_1 antagonist - [^{3}H]-Mepyramine to label H_1 receptors in mouse mammary gland and in mammary epithelial cells fraction. The saturation binding experiments showed the presence of specific binding sites for [^{3}H]-Mepyramine in whole mouse mammary gland in each: resting, pregnancy and lactation stage (*Table 1*). These binding sites were also found in freshly isolated and cultured mammary epithelial cells. Moreover, we observed a higher density of [^{3}H]-Mepyramine binding sites on MMEC compared to the whole gland preparations. The density of [^{3}H]-Mepyramine binding sites also tended to be higher in freshly isolated MMEC than in cultured epithelial cells (126 ± 52.9 vs 44.3 ± 6.4).

Specific binding sites for [^{3}H]-Tiotidine were not found in freshly isolated MMEC or in the membrane preparations from whole mammary gland.

We found specific binding of H_3 antagonist [^{3}H]-R(-)-α-Methylhistamine using membrane preparations from lactating mammary gland, indicating the presence of H_3 receptors in the mammary gland. The receptor affinity to [^{3}H]-R(-)-α-Methylhistamine was K_d=1.46 ±0.47 nM and estimated density was B_{max} = 3.7 ± 0.48 fmol/mg protein (data not shown).

Table 1. H_1 histamine receptor parameters in mouse mammary gland and isolated (cultured) epithelial cells at different functional stages of the gland. All values given are means ± SEM from single representative experiments.

Membrane preparations:	Binding sites Bmax [fmol/mg protein]	[^{3}H]-Mepyramine affinity Kd [nM]
Resting mammary gland	38.5±4.1	1.9±0.7
12th-15th day of pregnancy mammary gland	50.9±15.8	7.3±4.2
1st week of lactation mammary gland	28.7±4.1	4.7±1.4
2nd week of lactation mammary gland	20.0±9.5	7.5±6.0
Freshly isolated epithelial cells from 14th-18th day of pregnancy mammary gland	126.0±52.9	12.3±9.4
Primary epithelial cells culture derived from 14th-18th day of pregnancy mammary gland	44.3±6.4	7.3±2.3

DISCUSSION

Previous biochemical studies showed active histidine decarboxylase and its product - histamine in mammary glands and indicated major changes of this system during various physiological states (16). In this study we successfully targeted the cells responsible for these phenomena as well as providing evidence for histamine receptors involved. It is well known that mast cells are rich in histamine (3). Both HDC and histamine positive mast cells were localised immunohistochemically in mammary glands. It is evident from the subcellular analysis that HDC- positive immunoprecipitation was exhibited in granules. The latter observation agrees well with the recent findings of Japanese group (23). These authors studied the intracellular localization of the 74 and 53- kDa forms of HDC in RBL-2H3 cell line and demonstrated that posttranslationally processed HDC as 53 kDa form is originally localised in the endoplasmic reticulum and Golgi system and then moved and stored in the granules.

It should be mentioned that the mast cells found in lactating glands seem to have a different appearance than the cell population in glands from pregnant animals. The former cells are rather elongated and have an irregular shape, the latter have a regular and oval shape, are more compact giving stronger histamine signals.

Epithelial cells were considered as a second source of histamine. Indeed, both biochemical and immunohistochemical studies revealed the presence of histamine and histidine decarboxylase in primary epithelial cell cultures as well as the specific histamine receptors H_1 in the primary epithelial cell culture and H_1 and H_3 in mammary tissue. We found that epithelial cells isolated from resting, pregnancy and lactating mammary glands show different histamine synthesis profiles. The activity peak of HDC at the 12th day and plateau of enzyme activity recorded in the second and third decade of pregnancy was almost completely abolished after parturition and throughout first week of lactation. The distinct pattern of HDC activity changes in the cell culture model matches clearly the changes observed previously in mouse mammary gland during pregnancy and lactation (16). These data are thus in line with the suggestion that the epithelial histamine stimulates mammary gland growth and differentiation as well as function during lactation through a paracrine pathway and an autocrine loop (8, 9, 24).

To further characterize the mammary epithelial cells and HDC enzyme protein expression the complex immunochemical study was performed. The illustrations presented show HDC protein expression in cultured epithelial cells, especially in dividing and non differentiated cells. Moreover, it seems that the enzyme protein expression was relatively higher than in mammary gland tissue, in which the immunopositive epithelial structures were only found after the use of the more sensitive immunofluorescence method. It cannot be ruled out, that the positive effect on HDC regulation may have been due to the presence of glucocorticoids in the culture medium. Dexamethasone treatment up-regulates HDC expression in rat lungs (25) and in mastocytoma cells (26) via

glucocorticoid - responsive elements. However, the changes in HDC protein expression we found with the immunofluorescence method corresponded well to data obtained in biochemical study.

Saturation binding assays showed H_1 receptor presence in resting, pregnancy and lactation stages of mammary gland. The Kd values calculated for [^{3}H]-Mepyramine in our study are within a range of reported values (5-12 nM) obtained for rat and human mammary glands (10, 11). The affinity of [^{3}H]-Mepyramine to the H_1 receptor seems to be relatively higher in resting and 1 week of lactation mammary and it could be associated with HDC activity decrease and lower availability of histamine. The distribution of H_1 receptor in mammary gland is suggested to be largely due to epithelial cells as the density of H_1 receptors was significantly higher in isolated MMC population than in the whole gland (*Table 1*). A decrease in H_1 receptor expression under culture conditions was recorded. It is likely that glucocorticoid treatment (medium supplementation) could down-regulate H_1 and H_2 receptors expression, as was observed in cerebral endothelial cell cultures (6). Alternatively, this may suggest lower degree of differentiation of outgrowing cells or phenomenon of receptor internalisation during cell preparations. We report also the presence of H_3 receptors in mammary gland which may be associated with blood vessels and mast cells (27). In this context it is worth noting that the H_3 antagonist - FUB181 added positively to oxytocine-stimulated milk secretion from dairy goat mammary gland (15). No evidence was found for the presence of H_2 receptors in whole gland preparations or in epithelial cell fractions. This is in contrast to previously published work (10, 11) and the discrepancy may be explained by the differences in the assay conditions and the species studied (man, rat, mouse).

We propose that mouse mammary epithelial cells possessing specific HDC and bearing histamine receptors control the growth and differentiation of mammary gland in autocrine and paracrine fashion. Thus, histamine mostly of glandular origin is suggested to be involved in pregnancy mammary outgrowth as well as lactation.

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