Autoregulation of McA-RH7777 Hepatoma Cell Proliferation by Histamine H₃ Receptors

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ABSTRACT

Previous studies have suggested that histamine (HA) acts as an autocrine growth factor. We have explored the modulation of cell proliferation by HA using McA-RH7777 hepatoma cells. High L-histidine decarboxylase (HDC) expression and HA synthesis were found in McA-RH7777 cells. Whereas extracellular HA reached submicromolar concentrations, intracellular levels were very low, indicating that HA was secreted by the cells. McA-RH7777 cells also express H₃-receptor (H₃R) transcripts and proteins. Reverse transcriptase-polymerase chain reaction analysis detected only transcripts for the long isoform. Immunocytochemistry performed with a selective H₃R antibody showed that most cells were immunoreactive. H₃R binding sites (Bₚmax = -30 fmol/mg protein) were identified when [¹²⁵I]iodoproxyfan binding was displaced by the agonist imetit. High-affinity binding also occurred at cytochrome P450 enzymes. This binding was not inhibited by HA, H₃R antagonists, or by a nonimidazole H₃R antagonist but was displaced by imidazole H₃R antagonists or by ketoconazole, a imidazole-containing cytochrome inhibitor. HA induced proliferation of McA-RH7777 hepatoma cells. The absence of uptake system, its much higher potency at H₃Rs, and its low intracellular levels suggested that HA interacted with H₃Rs rather than cytochromes. In agreement, both imidazole H₃R antagonists, a nonimidazole H₃R antagonist, and the HDC inhibitor α-monofluoromethyl histidine increased cell proliferation (up to ~60%), revealing a H₃R-mediated inhibition by endogenous HA. Moreover, exogenous HA inhibited the increase induced by α-FMH or H₃R antagonists with a nanomolar potency. In conclusion, our findings show that HA regulates proliferation of McA-RH7777 hepatoma cells by interacting with autoinhibitory H₃Rs.

Besides its well established role in central neurotransmission, gastric acid secretion, and allergic and inflammatory reactions (Brown et al., 2001; Parsons and Ganellin, 2006), data accumulated over the years strongly suggest that histamine (HA) is also involved in tissue growth and cell proliferation. Initially, an important role of newly synthesized (“nascent”) HA in development and rapid tissue or tumor growth was proposed by Kahlson and co-workers in the 1960s (Kahlson and Rosengren, 1968). In agreement, depletion of HA levels suggested that HA interacted with H₃Rs rather than cytochromes. In addition, both imidazole H₃R antagonists, a nonimidazole H₃R antagonist, and the HDC inhibitor α-monofluoromethyl histidine increased cell proliferation (up to ~60%), revealing a H₃R-mediated inhibition by endogenous HA. Moreover, exogenous HA inhibited the increase induced by α-FMH or H₃R antagonists with a nanomolar potency. In conclusion, our findings show that HA regulates proliferation of McA-RH7777 hepatoma cells by interacting with autoinhibitory H₃Rs.

The targets and mechanisms involved in this autocrine effect of HA have remained unclear. It has been postulated that intracellular HA regulates cell growth by interacting with microsomal sites subsequently identified as cytochrome P450 enzymes, present in all cells but most abundant in the liver (Brandes et al., 1998; LaBella and Brandes, 2000). In addition, HA is well known to interact with four receptor subtypes (H₁, H₂, H₃, and H₄) belonging to the family of G protein-coupled receptors (GPCRs) (Hill et al., 1997; Hough, 2001). Activation of many GPCRs can stimulate cell proliferation, and GPCRs have recently emerged as crucial players in tumor growth (Dorsam and Gutkind, 2007). H₁ and H₂ receptors themselves have been found in multiple human and rodent tumors or tumoral cell lines, and various reports have suggested antitumoral properties of H₂-receptor antagonists (Bartholeyns and Bouclier, 1984; Rivera et al., 2000). The histamine H₄ receptor has also recently emerged as a therapeutic target for inflammatory and immune disorders.
that H3R and HDC mRNAs are coexpressed in various tissues at embryonic stages but not at adult stages. This indicated that H3 receptors are present on many dividing cells during development and may be activated by HA originating from the same cells (Héron et al., 2001). The embryonic liver contains not only very high levels of HDC and HA but also H3R mRNAs that are transiently expressed together and with a similar time course with HDC mRNAs, suggesting a H3R-mediated autocrine effect of HA on liver development (Héron et al., 2001).

In the present study, we have explored further the modulation of cell proliferation by HA using the McA-RH7777 hepatoma cell line. This cell line was previously shown to express HDC and synthesize HA (Brandes et al., 2002). We show that McA-RH7777 cells express native H3 receptors and that endogenous HA secreted from the same cells interact with H3 receptors to modulate their proliferation.

Materials and Methods

Cell Cultures. The McA-RH7777 cell line, derived from the Morris hepatoma 7777, and the COS-7 cell line, derived from CV-1 simian cell line, were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Les Ulis, France) and antibiotics (100 µg/ml streptomycin and 100 µg/ml penicillin) (Invitrogen). Cells were grown as monolayers in antibiotic- and serum-free medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Les Ulis, France). The obtained templates were amplified for 40 cycles (94, 57, and 72°C for 30 s each) using AmpliTaq Gold DNA polymerase (PerkinElmer, Courtaboeuf, France) and primers 1 and 2, based on the sequence of the fourth transmembrane domain and the third intracellular loop of the rat H3 receptor, respectively (Zhang et al., 2007). Whether it is also involved in regulation of tumor growth requires further work, but a recent study reported that H3-receptor activation inhibits cell proliferation in a breast cancer cell line (Medina et al., 2008).

The role of the H3 receptor (H3R) in cell proliferation is supported by the observation that activation of recombinant H3 receptors enhances mitogen-activated protein kinase activity or phosphorylation (Drutel et al., 2001; Héron et al., 2001). In addition, some studies have reported a modulation of cell proliferation in tissues by H3-receptor ligands (Morini et al., 2000; Morisset et al., 2000) (primer 1, 5’-TGCTGATGGGCTT-GCATCTCTAGTTGG-3’, and primer 2, 5’-CACATCTTCTCAGG-CTTCCACAGGATTGC-3’), or primers 3 and 4, based on the sequence of the rat HDC (primer 3, 5’-CATGCTGAGCGAG-CCTTGGAGCGCCGGC-3’, and primer 4, 5’-CATGCTGAGCGCTGAG-GACAAGTTAGCAGGC-3’). The β-actin cDNAs were obtained by PCR amplification of the first-strand cDNA for 25 cycles (94, 57, and 72°C for 30 s each) using primers 5 and 6 (primer 5, 5’-GATTGG-GGTATGAGTCAGGAAGAAA-3’, and primer 6, 5’-GTGCTCATGAC-GATGATGATGACCT-3’). PCR products were analyzed on a 2% agarose gel (Eurobio, Les Ulis, France).

Immunocytochemistry. COS-7 and McA-RH7777 cells maintained in culture were detached using Versene (EDTA; Invitrogen) or scraping, respectively, plated on glass microscope coverslips coated with collagen, and left to grow for 24 h. COS-7 cells were transiently transfected and used as controls. cDNA inserts coding for the full-length sequence of the rat H3R were ligated into the mammalian expression vector pCIneo (Promega, Madison, WI) and processed for immunocytochemistry after a 24-h incubation.

Cells were washed with phosphate-buffered saline (PBS) (0.1 M, pH 7.4) and fixed in a solution containing 3% paraformaldehyde in PBS for 40 min at room temperature. After three washes in PBS, cells were incubated for 10 min with 50 mM NH4Cl and permeabilized with 0.5% Triton X-100 in PBS for 1 min. After washing in PBS, cells were incubated in 20% normal goat serum for 1 h at room temperature. The normal goat serum at 10% in PBS was used for dilutions of primary and secondary antisera and different washes. Cells were incubated overnight at 4°C with the polyclonal rabbit anti-H3R antisera (Interchim, Montluçon, France) at 1:200. After three washes, cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (FluoProbes; Interchim) at 1:200 for 30 min at room temperature. After rinsing in PBS (3 × 10 min), cells were mounted on glass slides with Vectorshield (Vector Laboratories, Burlingame, CA), a DAPI-containing medium. Immunocytochemical labeling was analyzed by fluorescence microscopy (Axioplan 2 Imaging; Zeiss, Feldbach, Switzerland). No immunostaining was observed after omission of the primary or secondary antibody. Cell nuclei DAPI staining was used to confirm the presence of the cells.

Histamine Levels and Uptake. For the determination of endogenous HA levels, McA-RH7777 cells were cultured for 24 h in the presence or absence of o-phenanthroline (100 µM) or ciprofloxin (1 µM) in antibiotic- and serum-free medium in 24-well flat-bottomed microtiter plates. The medium and cells were treated for 30 min with 4% trichloracetic acid in ice, centrifuged for 10 min at 140g, and stocked at −20°C until use. Endogenous HA levels present in the medium and cell samples were measured in triplicate using an enzyme immunoassay kit (Immunotest, Marseille, France).

For uptake experiments, the cell culture (1 ml) was incubated for 5 min in antibiotic- and serum-free medium. Cells were then incubated for 10 min with [3H]histamine (GE Healthcare) (100 nM final concentration). After separation from the medium by decantation, the cells were washed twice in phosphate buffer and resuspended in 1 ml of the medium. The radioactivity present in cells and medium was determined by liquid scintillation counting in a β1410 Wallac counter (Pharmacia Biotech, Les Ulis, France).

Cell Proliferation Assays. To examine the effect of H3R ligands on McA-RH7777 cell proliferation, cells were harvested in exponential growth phase, washed three times with phosphate buffer, pH 7.4, adjusted to 2 × 10^5 cells/ml, and cultured for 24 h in 96-well flat-bottomed microtiter plates in antibiotic- and serum-free medium.
containing, when required, the various drugs. The number of living McA-RH7777 cells was then as assessed by a quantitative colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and an electron coupling agent (phenazine ethosulfate) (CellTitre 96 AQuinnus One Solution Reagent; Promega). Assays were performed by the addition (20 μl) of the One Solution Reagent directly to culture wells, incubation for 4 h at 37°C, and reading of absorbance at 490 nm with a 96-well plate reader (MRXII; Dynex Technologies, Chantilly, VA). Data were expressed as the percentage of the number of living cells compared with control (nontreated) cells.

In another sets of experiments, cells were first cultured for 24 h in the presence of 100 μM α-FMH or 1 μM ciproxifan in serum- and antibiotic-free medium in 96-well flat-bottomed microtiter plates. Cells were then harvested and further cultured for 24 h in the same α-FMH- or ciproxifan (CPX)-containing medium with, when required, increasing concentrations of histamine or R- or S-α-methylhistamine. The number of living McA-RH7777 cells was then assessed as described above.

Apoptosis Assays. The number of apoptotic cells was assessed using a quantitative terminal deoxynucleotidyl transferase dUTP nick-end labeling method (In Situ Cell Death Detection Kit, AP; Roche Applied Science, Meylan, France). After 24-h incubation in 96-well flat-bottomed microtiter plates, cells were fixed overnight at 4°C by the addition of 4% paraformaldehyde, final dilution. After three washes in PBS, cells were then incubated with the terminal deoxynucleotidyl transferase dUTP nick-end labeling reaction mixture that contains terminal deoxynucleotidyl transferase and fluorescein-dUTP. The labeling incorporated at the damaged sites of the DNA was then revealed by an anti-fluorescein antibody conjugated with the reporter enzyme alkaline phosphatase. After washing to remove unbound enzyme conjugate, the alkaline phosphatase retained in the immune complex was visualized by the substrate reaction. Quantitative detection of apoptotic cells was applied by light microscopy. Living cells were refringent, whereas apoptotic dead cells were dark.

Analysis of Data. The total curves were analyzed with an iterative least-squares method derived from that of Parker and Waud (1971). Computer analysis was performed by nonlinear regression using a one-site cooperative model. The method provided estimates for K_d and B_{max} values ([125I]iodoproxyfan), IC_{50} values, EC_{50} values, and pseudo-Hill coefficients (n_H). In cell proliferation experiments, K_i values of the antagonists/inverse agonists were evaluated from their half-maximal concentration (IC_{50}). The relationship, K_i = IC_{50}(1 + S/EC_{50}), was used (Cheng and Prussoff, 1973), assuming that the compounds were acting as competitive H_3R antagonists against endogenous HA released in the medium. The concentration of endogenous HA (S) was assumed to be in each case the same as that determined at the end of a 24-h incubation without compounds, i.e., ~230 nM. The concentration required for the half-maximal effect of endogenous HA (EC_{50}) was considered to be the same as that determined for exogenous HA in the presence of ciproxifan, i.e., 5.1 ± 0.4 nM. Protein contents were determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Radiochemicals and Drugs. [125I]Iodoproxyfan (2000 Ci/mmol) was prepared as described previously (Krause et al., 1997). [3H]Histamine (51 Ci/mmol) was from GE Healthcare. The drugs and their sources were as follows: ciproxifan, thioperamide, and BF2.649 were from Bioprojet (Paris, France); clobenpropit was from Tocris (Bristol, UK); imetit was provided by C. R. Ganellin (University College, London, UK); FUB 349, FUB 465, and R- and S-α-methylhistamine were provided by W. Schunack (Freie Universität, Berlin, Germany); and histamine and ketoconazole were from Sigma-Aldrich (St Louis, MO). All other chemicals were from commercial sources and were of the highest purity available.

Results

[125I]Iodoproxyfan Binding. Imidazole H_3R inverse agonists inhibited the binding of [125I]IPX (450 pM) to McA-RH7777 rat cell membranes. Total binding (~250 fmol/mg protein) was completely inhibited by CPX, thioperamide, and clobenpropit with IC_{50} values of 44 ± 1, 27 ± 1, and 162 ± 1 nM, respectively, and pseudo-Hill coefficients were not significantly different from unity (Fig. 1A). A slight but nonsignificant decrease of [125I]IPX binding (by ~5–10%) tended to be induced by the lowest concentrations (0.01–1 nM) of ciproxifan (Fig. 1A). BF2.649, a nonimidazole H_3R inverse agonist (Ligneau et al., 2007), did not significantly decrease the total binding of [125I]IPX, even when it was added at a high concentration (100 μM). Histamine and the H_3R agonist, imetit and R-α-MeHA, also failed to significantly inhibit total binding, although a slight but nonsignificant inhibition (by ~10–20%) was observed at the highest concentrations tested (10–100 μM).

The total binding observed to cell membranes in the presence of 20 or 300 pM [125I]IPX (29 ± 1 and 117 ± 7 fmol/mg protein, respectively) was also inhibited in a dose-dependent manner (Fig. 1B).
manner by ketoconazole, a imidazole-containing broad-spectrum cytochrome P450 inhibitor (Higashi et al., 1987) (Fig. 1B). Ketoconazole progressively inhibited the binding with \( IC_{50} \) values of 0.12 ± 0.01 and 0.37 ± 0.01 \( \mu \)M at 20 and 300 \( \mu \)M \([^{125}I]\)IPX, respectively.

When defined as that inhibited by imetit (6 \( \mu \)M), \([^{125}I]\)IPX-specific binding to McA-RH7777 cell membranes was saturatable (Fig. 2). Analysis of the data using an one-site cooperative model indicated that the Hill coefficient was not significantly different from unity, with a \( K_D \) value of 29 ± 7 \( \mu \)M and a \( B_{\text{max}} \) value of 27 ± 3 fmol/mg protein. Scatchard analysis of saturation binding data also disclosed a single population of sites with a \( K_D \) value of 35 ± 11 \( \mu \)M and a \( B_{\text{max}} \) value of 26 ± 1 fmol/mg protein (Fig. 2). Specific binding represented approximately 10% of total binding at 180 \( \mu \)M \([^{125}I]\)IPX.

**RT-PCR Analysis of H\textsubscript{3}R and HDC Gene Transcripts.** The existence of H\textsubscript{3}R gene transcripts was investigated by PCR analysis of cDNAs from McA-RH7777 cells using specific primers 1 and 2. The gel-resolved PCR products comprised two fragments of ~320 and ~520 base pairs in length (Fig. 3). Sequence analysis of these products confirmed that the largest fragment corresponded to the longer form (H\textsubscript{3}(445)) of the rat H\textsubscript{3}R (Lovenberg et al., 2000; Morisset et al., 2000). In contrast, the smallest fragment did not correspond to any H\textsubscript{3}R isoform and was, therefore, generated by nonspecific amplification (Fig. 3).

A very high HDC mRNA expression was observed in McA-RH7777 cells. After amplification with specific primers 3 and 4, a single cDNA fragment was obtained, with a length corresponding to that expected from the rat HDC nucleotide sequence (Joseph et al., 1990) (Fig. 3).

**H\textsubscript{3}R Immunolabeling.** Fluorescence microscopy performed with a selective H\textsubscript{3}R antibody showed that the vast majority of McA-RH7777 cells, if not all, were immunoreactive for the H\textsubscript{3}R (Fig. 4a). However, the intensity of the observed immunostaining remained moderate and was lower than that observed following transient expression of the receptor in COS(H\textsubscript{3}R) cells, a cell line used as a positive control (Fig. 4b). No labeling was observed with the corresponding mock cells identified with the DAPI staining (Fig. 4c) or when immunocytochemistry was carried out on cells by omitting the primary or secondary antibodies (data not shown).

**Fig. 2.** Saturation of specific \([^{125}I]\)IPX binding to McA-RH7777 cell membranes. Membranes were incubated for 60 min at 25°C with \([^{125}I]\)IPX in increasing concentrations. Specific binding was defined as that inhibited by 6 \( \mu \)M imetit. The inset shows the Scatchard transformation of the data. Each point represents the mean of triplicate determinations from a single experiment, which was repeated twice with similar results.

**Fig. 3.** RT-PCR analysis of H\textsubscript{3}R and HDC gene transcripts in McA-RH7777 cells. Total RNAs from cells were digested with RNase-free DNase I to avoid any amplification of residual genomic DNA and subjected to reverse transcription. The obtained templates were amplified for 40 cycles (H\textsubscript{3}R and HDC) (94, 57, and 72°C for 30 s each) with selective primers. \( \beta \)-Actin mRNAs were amplified for 25 cycles. No signals were observed when the templates were omitted and replaced by water.

**Fig. 4.** Immunolabeling of H\textsubscript{3}R immunoreactivity in McA-RH7777 cells. McA-RH7777 cells (a), Cos(H\textsubscript{3}R) cells (b) as the positive control, and the corresponding mock cells (c) as the negative control were fixed with 3% paraformaldehyde, washed, and processed for immunolabeling using a polyclonal rabbit anti-H\textsubscript{3}R antiserum as the primary antibody, and a goat anti-rabbit IgG was conjugated to Alexa Fluor 488 as the secondary antibody. After rinsing in PBS, cells were mounted with Vectashield, a mounting medium with DAPI. The immunostaining was observed by fluorescence microscopy. Magnifications, \( \times 10,000 \) (1 cm = 10 \( \mu \)m).
Histamine Levels and Uptake. A submicromolar concentration of endogenous histamine was measured in the medium of McA-RH7777 cells cultured for 24 h. This concentration was dramatically reduced when the cells were cultured for the same time in the presence of 100 μM α-FMH, a specific inhibitor of HDC (Kollonitsch et al., 1978), but remained unchanged after culture of the cells with 1 μM CPX (Table 1). The endogenous histamine level found inside the cells was only in the nanomolar range, i.e., much lower than that found in the culture medium (Table 1). This intracellular histamine level was not significantly modified after incubation of cells with α-FMH or CPX (Table 1). After a 10-min incubation of McA-RH7777 cells in the presence of 100 nM [3H]HA, no significant uptake of [3H]HA (<1%) could be detected within the cells (data not shown).

Effect of H₃R Ligands on McA-RH7777 Cell Proliferation. Cells were incubated for 24 h with increasing concentrations (up to 100 μM) of histamine or R-α-MeHA. The two agonists failed to significantly modify the number of living McA-RH7777 cells (data not shown).

The number of living McA-RH7777 cells was then determined after incubation for 24 h with increasing concentrations of H₃R antagonists/inverse agonists. All of the compounds tested, including the nonimidazole inverse agonist BF2.649 (Ligneau et al., 2007), increased cell proliferation in a concentration-dependent manner. Their potencies were all determined after incubation for 24 h with increasing concentrations (up to 100 μM) of histamine or R-α-MeHA. The two agonists failed to significantly modify the number of living McA-RH7777 cells (data not shown).

The absence of effect by agonists and the increase induced by antagonists/inverse agonists could suggest a significant occupation of H₃Rs by endogenous HA levels. To further test this hypothesis, McA-RH7777 cells were incubated with α-FMH. After incubation of the cells for 24 h with 100 μM α-FMH, the number of living cells was increased by 32%/H9251 (±14%) (Fig. 5; Table 2). CPX did not change the number of apoptotic cells (~5%; data not shown).

A very similar increase (33% ± 2%) was induced by α-FMH when the cells were incubated with the drug (100 μM) for two successive 24-h periods (Fig. 6). When exogenous histamine was added in various concentrations to the culture medium during the second period, the increase in cell proliferation induced by 100 μM α-FMH was progressively reduced by approximately half (14% ± 4%). Analysis of the concentration-response curve led to an EC₅₀ for HA of 2.3 ± 1.2 nM (Fig. 6). In other sets of experiments, cells were cultured in the same conditions (two successive 24-h periods), but in the presence of 1 μM CPX instead of α-FMH. The increase in cell proliferation induced by CPX (+56% ± 5%) was again progressively reduced by exogenous HA with an EC₅₀ value, determined from analysis of the total curve, of 5.1 ± 0.4 nM (Fig. 6). However, the inhibitory effect of HA was much more pronounced, the increasing effect of CPX being totally suppressed. At the highest concentrations of histamine tested (30 nM–10 μM), a decrease in the number of living cells tended to be observed compared with controls (absence of CPX) (~19% ± 3%), but this decrease remained statistically nonsignificant. In other sets of experiments, CPX-induced proliferation was inhibited not only by HA (EC₅₀ of 3.1 ± 0.6 nM) but also in a stereoselective manner by α-MeHA. The R-isomer fully inhibited proliferation with an EC₅₀ of 1.1 ±

Table 1

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<thead>
<tr>
<th>Treatment</th>
<th>Histamine Levels</th>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>None</td>
<td>231 ± 20</td>
</tr>
<tr>
<td>α-FMH</td>
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<tr>
<td>Ciproxifan</td>
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Table 2

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<th>Drugs</th>
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<tr>
<td></td>
<td>μM</td>
<td>%</td>
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<tr>
<td>Ciproxifan</td>
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<td>63 ± 3</td>
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<tr>
<td>BF2.649</td>
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<td>49 ± 4</td>
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<tr>
<td>Thioperamide</td>
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<tr>
<td>Clobenpropit</td>
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<td>FUB 349</td>
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<td>31 ± 3</td>
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<tr>
<td>FUB 465</td>
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Fig. 6. Effect of histamine on the increase in cell proliferation induced by 1 mM CPX (■) or 100 nM α-FMH (○). Cells were first cultured for 24 h in the presence of 1 mM CPX or 100 nM α-FMH. They were then harvested and further cultured for 24 h with exogenous histamine in increasing concentrations in the presence of 1 mM CPX or 100 nM α-FMH. The number of living cells was then assessed by a quantitative colorimetric method. Results are expressed as the percentage of the number of living cells in the absence of any drug. Each point represents the mean ± S.E.M. from six separate experiments with quadruplicate determinations.

Fig. 7. Effect of histamine (■), R-α-methylhistamine (○), and S-α-methylhistamine (▲) on the increase in cell proliferation induced by 1 mM CPX. Cells were first cultured for 24 h in the presence of 1 mM CPX. They were then harvested and further cultured for 24 h with the agonists in increasing concentrations in the presence of 1 mM CPX. The number of living cells was then assessed by a quantitative colorimetric method. Results are expressed as the percentage of the number of living cells in the absence of any drug. Each point represents the mean ± S.E.M. from six separate experiments with quadruplicate determinations.

Discussion

The high HDC expression and HA synthesis in McA-RH7777 rat hepatoma cells are reminiscent of the high but transient HDC and histamine contents found in the rat embryonic liver (Kahlson and Rosengren, 1968; Héron et al., 2001). The submicromolar levels of histamine in the culture medium are consistent with those reported in the same cell line (Brandes et al., 2002). The decrease of these levels induced by α-FMH, a selective HDC inhibitor (Kollontisch et al., 1978), confirmed that they emanate from HA synthesis within the cells. However, the intracellular levels were very low, showing that HA is totally secreted in the external medium. This finding and the absence of [3H]histamine uptake indicate that no reuptake system is present in these cells for histamine.

RT-PCR analysis, immunocytochemistry, and [125I]iodoproxyfan binding all demonstrated moderate levels of H3Rs on McA-RH7777 cells. It is interesting that, among the H3R rat isoforms (Hancock et al., 2003), only the long (nondeleted) H3(445)R, H3(413)R, and H3(397)R isoforms were amplified in the embryonic (but not adult) liver (Héron et al., 2001). Moreover, a saturable H3R population was identified when [125I]iodoproxyfan binding was displaced by the agonist imetit, with a KD of 35 nM (Héron et al., 1994).

In addition to this H3R binding, the huge density of cytochrome P450 enzymes within liver cells, including hepatoma cells such as McA-RH7777 cells (Brandes et al., 2002), accounts for a much larger (by ~10-fold) high-affinity binding, clearly occurring to a non-H3R component. This non-H3R binding was inhibited with high affinity by H3R imidazole antagonists but with low affinity by H3R agonists (including HA). It was already observed in rat brain or liver with [125I]iodoproxyfan itself (Ligneau et al., 1994) or other H3R radioligands (Alves-Rodrigues et al., 1996, 1998) and was suggested to correspond to P450 enzymes. Our binding data strongly support this hypothesis. First, the non-H3R binding was fully displaced by ketoconazole, an imidazole-containing broad-spectrum cytochrome P450 inhibitor, with a consistent submicromolar potency (Higashi et al., 1987; Yan et al., 2002). Second, it was also fully inhibited by the three imidazole-containing compounds, ciproxifan, thioperamide, and clobenpropit, with submicromolar potencies similar to those found at adrenal P450 enzymes (LaBella et al., 1992; Yang et al., 2002) but two orders of magnitude lower than their affinities at rat H3Rs (Stark et al., 2001). Although H3R agonists contain the imidazole ring, which inhibits P450 isozymes through coordination with their heme moiety, they lack the lipophilic side chain present in antagonists. Consistent with their resulting weak potency at liver microsomes (Brandes et al., 1998) and adrenal P450 enzymes (LaBella et al., 1992; Yang et al., 2002), HA and H3R agonists failed to affect the non-H3R binding at concentrations up to 0.1 mM. As already reported (Ligneau et al., 1994), the agonist imetit was allowed to discriminate the specific (H3R) from the non-specific (non-H3R) binding, although the latter was much higher. At last, nonimidazole H3R antagonists are much more potent that was found again in the nanomolar range (data not shown).
weaker inhibitors of P450 enzymes than imidazole compounds, a property that increases their tolerability (Zhang et al., 2005) and clinical interest for the treatment of central disorders (Hancock, 2006). As expected for P450 enzymes, the nonimidazole H3R antagonist BF2.649 (Ligneau et al., 2007) failed to inhibit [125I]iodoproxyfan binding, even at high concentrations.

Its binding potency at H3Rs being in the nanomolar range (Hill et al., 1997) and its extracellular levels reaching submicromolar concentrations, HA secreted by McA-RH7777 cells is expected to occupy most H3Rs present at their surface. Whether endogenous HA can also interact with cytochromes within the cells seems less likely. The intracellular HA concentrations after a 24-h culture period were in the nanomolar range, i.e., at least 1000-fold lower than those required to interact with rat cytochromes (Brandes et al., 1998). Vice versa, these very low intracellular HA levels are not consistent with an HA pool tightly bound to cytochromes. However, the high HDC activity may yield transiently high enough HA levels to occupy P450 enzymes at some set times.

High synthesis and rapid diffusion of histamine during cell proliferation have been observed in other cells and tissues undergoing rapid growth, including hepatoma (Kahlson and Rosengren, 1968; Bartholeyns and Bouclier, 1984; Medina et al., 1999). In addition, this “nascent” HA could regulate cell proliferation and tumor growth, raising the hypothesis that HA was acting as an autocrine growth factor. Consistent with such an autocrine function, we show in this study that endogenous, as well as exogenous, HA inhibits McA-RH7777 cell proliferation.

Among the putative targets of HA in this autoregulatory effect, P450 enzymes themselves regulate cell proliferation by controlling the levels of growth regulatory factors (Nebert, 1991). Moreover, the interaction of HA with cytochromes P450 has been implicated in cell growth modulation (Brandes et al., 1998; LaBella and Brandes, 2000). However, our data do not support such an interaction. First, as discussed above, the low potency of HA at cytochromes and its low intracellular levels indicate that the autoinhibition revealed through synthesis inhibition by α-FMH does not result from an interaction of endogenous HA with P450 enzymes. Second, in the absence of any uptake system, exogenous HA is not expected to enter the cells and reach high enough concentrations to interact with P450 enzymes. At last, the nonimidazole H3R antagonist BF2.649 (Ligneau et al., 2007) strongly increased cell proliferation but failed to inhibit the non-H3R (cytochrome) [125I]iodoproxyfan binding.

The role of autoinhibitory H3Rs in the inhibitory effect of HA is clearly predominant, if not exclusive. In agreement, exogenous HA inhibited cell proliferation with a nanomolar potency, a finding confirming that the modulation induced by endogenous HA is mediated by H3Rs.

In conclusion, the present study shows that H3Rs act as autoinhibitory receptors on proliferation of a tumor-derived cell line. Such an autoinhibitory property was already reported for H3 autoreceptors inhibiting histamine neuron activity in the brain (Arrang et al., 1983) and non-neuronal H3Rs inhibiting histamine release from rat gastric enterochromaffin-like cells (Prinz et al., 1993), suggesting that this is a general feature of H3Rs. As previously proposed (Kahlson and Rosengren, 1968; Bartholeyns and Bouclier, 1984; Medina et al., 1999; Rivera et al., 2000), drugs modulating the effect of histamine on tumor growth might be useful as adjuvant agents in cancer chemotherapy. The presence of functional H3Rs in tumors, as well as their physiological role on cell proliferation, remains to be established by further studies. If it is observed in vivo, such an H3R-mediated autoinhibition would raise a potential interest of H3R agonists for cancer prevention and treatment.

References


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