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MOUSE P19 EMBRYONAL CARCINOMA CELLS EXPRESS FUNCTIONAL HISTAMINE H_1-RECEPTORS

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In the present study, we have investigated the response in P19 embryonal carcinoma (EC) cells to histamine. We show that these cells, that resemble the pluripotent cells of an early mouse embryo, respond to histamine addition by a transient increase in intracellular Ca^{2+}. The response is stereoselectively inhibited by the enantiomers of the H_1-receptor antagonists chlorpheniramine and cicletanine. [^{3}H]-mepyramine was found to bind with high affinity (K_d 4 nM) to a membrane preparation of P19 EC cells. The profile of these binding sites corresponded well with the results of the Ca^{2+} measurements. A high affinity [^{3}H]-mepyramine binding site was also identified on intact cells. These data demonstrate that embryonal carcinoma cells express functional histamine H_1-receptors and suggest that histamine might act as a regulatory factor in the early development of the mouse embryo.

Histamine is an ubiquitous compound, which acts as a neurotransmitter in the brain and as a local hormone in many peripheral tissues (1,2,3). In cultured cells, histamine has been shown to induce changes in cell shape (4), chemotaxis (5), expression of cytokines (6,7) and their receptors (8), or cell growth (5,9,10,11). To date three receptor subtypes, known as the H_1-, H_2-, and H_3-receptors have been distinguished on the basis of their pharmacology (1,2,12). Recently the H_1- (13) and the H_2-receptor (14,15) have been cloned. Apart from these cell surface receptors an intracellular receptor, named the H_{1C}-receptor (16), has been suggested, which is pharmacologically different from the H_1-, H_2-, and H_3-receptors. The H_{1C}-receptor has been implicated in in vivo tumour growth (17), but further investigations are needed to see whether it is a functional histamine receptor.

In this study we present data indicating that histamine might act also as a regulatory factor in early mammalian embryonic development. In initial experiments we found that

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histamine is able to increase the intracellular Ca^{2+} concentration in a variety of cultured embryonal carcinoma (EC) and embryonic stem (ES) cells. EC and ES cells are frequently used as a model to study differentiation in the early mouse embryo in view of their antigenic and biochemical similarity with cells of the inner cell mass of blastocyst stage embryos (18). We used the P19 EC cell line (19) to characterize the response to histamine in more detail.

The initial observation that histamine induces a Ca^{2+} response in these cells suggests a role of the histamine H_1-receptor, which activates the phospholipase-C pathway upon stimulation by histamine leading to a release of Ca^{2+} from intracellular stores (4,5,20). We therefore performed fluorometric Ca^{2+} measurements of P19 EC cells labelled with Fura-2, taking the induction of a Ca^{2+} response by histamine as an indication of the presence of functional H_1-receptors. In order to demonstrate the presence of H_1-receptors directly on P19 EC cells, we performed binding studies, using the H_1-antagonist [^{3}H]-mepyramine as a radioligand (21). In both series of experiments, the results were verified pharmacologically by testing the degree of stereoselectivity of the enantiomers of the H_1-antagonists chlorpheniramine and cicletanine, which has already proven to be a valuable tool in evaluating the receptor specificty of the observed effects (22,23).

MATERIALS AND METHODS

Cell culture: P19 EC cells were cultured as previously described (24). Two days before experimentation, the cells were plated at a density of 15000 cells per cm^2, to yield subconfluent cultures for experiments 2 days later.

[^{3}H]-mepyramine binding to membranes: A membrane fraction of P19 EC cells was prepared as described by Nakahata et al. (25). For the saturation binding experiments, membranes (100-200 μg protein) were incubated with increasing concentrations of [^{3}H]-mepyramine (1-30 nM) for 10 minutes at 30°C in a total volume of 400 μl of 10 mM HEPES with 5 mM $MgCl_2$ (pH 7.4). Non-specific binding was determined in the presence of the H_1-receptor specific antagonist (-)cicletanine (3μM). For the displacement studies, 3 nM [^{3}H]-mepyramine was incubated with increasing concentrations of the indicated drugs. The incubation was started by the addition of the membranes, and stopped by rapid dilution with 3 ml cold buffer (50 mM Tris, 110 mM NaCl, 5 mM $MgCl_2$, pH 7.4) and filtration through Whatman GF/C filters. The radioactivity retained on the filters was determined by liquid scintillation counting.

[^{3}H]-mepyramine binding to intact cells: P19 EC cells were cultured in 6 well plates (Costar) as described above, and treated with serum-free medium for one hour before labelling. The cells were then incubated for one hour with 4 nM [^{3}H]-mepyramine in HEPES buffered DMEM at 4°C in the presence of either unlabelled mepyramine (26) or cicletanine. The reaction was stopped by three washes with PBS to remove unbound ligand, and cell protein was precipitated with 1 ml 0.2 M NaOH. The bound radioactivity was determined for the individual wells by liquid scintillation counting. In each experiment the conditions were tested in triplicate.

Protein assay: Protein concentrations were determined according to Bradford (27), using bovine serum albumin as a standard.

Ca^{2+} measurements: P19 EC cells were grown on tissue culture plastic coverslips, and loaded with 10 μM Fura-2/AM for 30-45 minutes at 33°C in HEPES buffered saline

(HBS) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose and 0,2% BSA, pH 7.3. During the experiment, the cells were maintained in HBS at 33°C. The measurements were carried out with a fluorescence microscope focussing on a group of 8-12 cells, and a SPEX dual wavelength fluorometer (SPEX Industries Inc, N.Y., U.S.A.) as excitation source. The emission fluorescence was monitored by computer software (SPEX 3000 DM), and corrected for the background fluorescence as determined from unlabelled cells. Subsequently, the intracellular Ca^{2+} concentration was calculated according to the method of Grynkiewicz (28). Minimal (F_{min}) and maximal fluorescence (F_{max}) were determined as previously described (20).

Chemicals: Histamine dihydrochloride and mepyramine (maleate salt) were obtained from Sigma Chemical Company Ltd. (St. Louis, MO, U.S.A.), Fura-2 acetoxymethylester (Fura-2/AM) from Molecular Probes (Eugene, OR, U.S.A.), and [pyridinyl-5-^{3}H]pyrilamine ([^{3}H]-mepyramine) from Amersham (Buckinghamshire, U.K.). The enantiomers of cicletanine were kind gifts from the Henri Beaufour Institute-IPSEN Laboratories, France. Dimaprit dihydrobromide was produced at the Department of Pharmacochemistry, Free University, Amsterdam, The Netherlands (29). Thioperamide and (R)α-methylhistamine were kindly donated by J.C. Schwartz, Centre Paul Broca de l'INSERM, Paris, France. Tiotidine was obtained from Imperial Chemical Industries.

RESULTS AND DISCUSSION

H_1-receptor mediated Ca^{2+} response in P19 EC cells

Upon stimulation with histamine, Fura-2 loaded P19 EC, F9 EC, ES 5, and D3 ES cells showed a transient rise in the intracellular Ca^{2+} concentration. **Figure 1A** demonstrates the Ca^{2+} response to histamine in P19 EC cells. The response was monophasic and not affected by the absence of extracellular Ca^{2+} (HBS supplemented with 4 mM EGTA; data not shown), indicating that the Ca^{2+} rise is due to release of Ca^{2+} from intracellular stores. Furthermore, the response was not inhibited by pretreatment of the cells with the G-protein inhibitor pertussis toxin (data not shown). This shows that the receptor couples to the class of pertussis toxin insensitive G-proteins, as has also been reported for the H_1-receptor on human 1321N1 astrocytoma cells (25). The cells respond to histamine in a dose-dependent manner (**figure 1B**) as determined from the peak of the response relative to the steady state Ca^{2+} concentration (93 $\pm$ 7 nM (mean $\pm$ s.e.m., n=15). The maximal and the half-maximal responses were found at 100 μM and 20 μM histamine, respectively. This result is in agreement with the dose-dependence of the Ca^{2+} response to histamine in endothelial cells (4).

To test whether the H_1-receptor mediates the response to histamine, we examined the effect of H_1-antagonists. **Figures 1C** and **1D** show the Ca^{2+} response to 100 μM histamine in the presence of cicletanine. The chiral H_1-antagonist cicletanine (31) showed a clear stereoselectivity at concentrations from 1 μM to at least 15 μM, at which (-)cicletanine was completely inhibitory (**1C**) and the inactive enantiomere (+)cicletanine had no effect (**1D**). These effects correspond to the reported stereospecific inhibition by cicletanine of the Ca^{2+} response to histamine in HeLa cells (20), in guinea-pig aorta vascular smooth

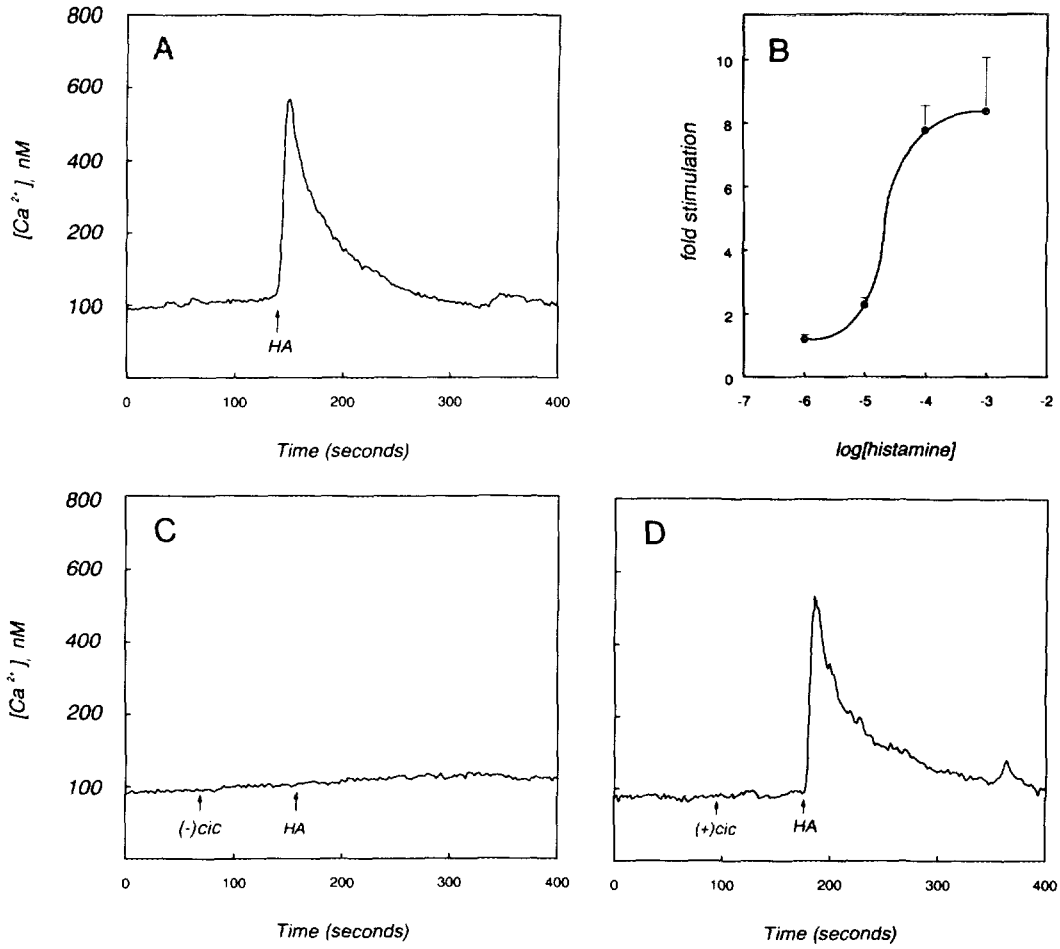

FIGURE 1. **A** Trace of the intracellular Ca^{2+} concentration calculated from the fluorescence ratio (340 nm/380 nm) of Fura-2 loaded P19 EC cells showing the response to 100 μM histamine. **B** Dose-response relationship showing the concentration dependent induction of a Ca^{2+} response by histamine, calculated at the peak of the response relative to the steady state Ca^{2+} level. **C** The response to 100 μM histamine in the presence of the H_1-receptor antagonist (-)cicletanine (15 μM) and **D** its inactive isomer (+)cicletanine (15 μM).

muscle cells (31), and the histamine induced contraction of guinea-pig ileum (30). Similar results were obtained for the enantiomers of chlorpheniramine, although this compound showed a slightly weaker stereospecificity compared to cicletanine (data not shown).

We also tested the effect of H_2-receptor (dimaprit (100 μM) and tiotidine (1 μM)), and H_3-receptor ligands ((R)α-methylhistamine (1 μM) and thioperamide (1 μM)) on the intracellular Ca^{2+} concentration. Neither the H_2-receptor nor the H_3-receptor selective ligands affect intracellular Ca^{2+} concentrations, and their presence did not affect the Ca^{2+} response upon the addition of histamine, indicating that the response to histamine is not mediated by the H_2- or the H_3-receptor in P19 EC cells (data not shown).

[^{3}H]-mepyramine binding to membranes of P19 EC cells

In the attempt to demonstrate the presence of receptors for histamine directly on embryonal carcinoma cells, we characterized the binding of the radiolabelled H_1-receptor antagonist [^{3}H]-mepyramine to undifferentiated P19 EC cells. In initial experiments, we used a membrane fraction to analyze the binding of the radioligand. At 30°C maximum specific binding was achieved within 5 minutes and lasted for at least 45 minutes (data not shown).

Saturation experiments with (-)cicletanine (3 μM) to define the non-specific binding revealed the presence of a saturable [^{3}H]-mepyramine binding site (**figure 2A**). Scatchard analysis of the specific binding data revealed a single high affinity [^{3}H]-mepyramine binding site with a K_d-value of 4.3 $\pm$ 0.5 nM (mean $\pm$ s.e.m., n=6), and a maximal binding capacity of 113 $\pm$ 32 fmol/mg protein (mean $\pm$ s.e.m., n=6). The K_d-value of [^{3}H]-mepyramine on membranes of P19 EC cells appears to be in the same range as that in mouse brain (2-7 nM) (32) and human 1321N1 astrocytoma cells (25). To address the question whether the high affinity [^{3}H]-mepyramine binding sites on the P19 EC cell membranes represent H_1-receptors, we used the stereoselective isomers of H_1-receptor specific antagonists to distinguish the receptor from previously identified high affinity non-receptor binding sites (23,33). **Figure 2B** shows a typical example of the stereospecific

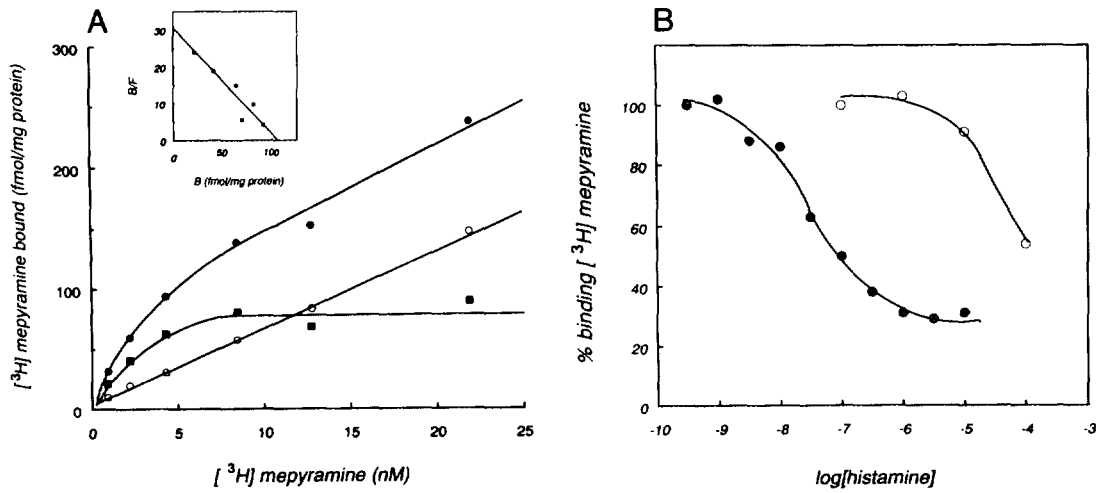

FIGURE 2. **A** Saturation experiment of [^{3}H]-mepyramine binding to membranes of P19 EC cells. The non-specific binding was determined in the presence of 3 μM (-)cicletanine (open circles). The specific binding (filled squares) was found by substracting the non-specific binding from the total binding (filled circles). The inset shows the transformation of the data into a Scatchard plot. **B** Dose dependent inhibition of the binding of 3 nM [^{3}H]-mepyramine to P19 EC membranes by the H_1 selective antagonist (-)cicletanine (filled circles) and by its stereoisomer (+)cicletanine (open circles). The data of **A** and **B** are typical for six experiments.

displacement by (-) and (+)cicletanine. A complete displacement curve for (+)cicletanine could not be obtained as the limit of solubility was reached. Chlorpheniramine was tested in a similar experiment (data not shown). From the IC_{50} values, deduced from the displacement curves, the respective K_i-values were calculated. The active H_1-antagonist d-chlorpheniramine (K_i 21.1 $\pm$ 4.9 nM) was as potent as (-)cicletanine (K_i 23.4 $\pm$ 3.2 nM) in inhibiting the [^{3}H]-mepyramine binding, while its isomer l-chlorpheniramine was only active at a 15-fold higher concentration (K_i 321 $\pm$ 53 nM). The K_i-values of these isomers on P19 EC cells correspond well with the previously reported results on the mouse brain (32), although the l-isomer had a slightly higher activity in P19 EC cells. For cicletanine we found an even larger difference (>1000 fold) between the two enantiomers. Its K_i-values are in good agreement with our findings on 1321N1 astrocytoma cells (R. Leurs, unpublished results), which contain H_1-receptors (25). The inhibition by the two H_1-receptor antagonists of the functional response and the binding of [^{3}H]-mepyramine to membranes of P19 EC indicate that cicletanine is the most discriminative antagonist in showing the highest degree of stereospecificity in these cells. Finally, binding of [^{3}H]-mepyramine to P19 EC membranes was not affected by the H_2-receptor antagonist tiotidine or the H_3-receptor antagonist thioperamide at concentrations up to 50 μM (data not shown).

[^{3}H]-mepyramine binding to intact P19 EC cells

Having identified the high affinity H_1-receptors on membrane preparation of P19 EC cells, we also established conditions for determining binding of [^{3}H]-mepyramine to intact cells. Generally, this approach would be a valuable tool for studying H_1-receptor expression in various embryonal carcinoma and embryonic stem cell lines, since fewer cells are required than for binding to isolated membranes. However, binding studies on intact cells using [^{3}H]-mepyramine have been hampered by high levels of accumulation of the radioligand into intracellular compartments, as well as binding to high affinity, non-receptor sites (34). To reduce the intracellular accumulation of [^{3}H]-mepyramine, we incubated the cells at 4°C. The total binding reached a plateau within 45 minutes (results not shown), so that for subsequent experiments the cells were incubated for 60 minutes. **Figure 3** shows a Scatchard plot of the binding of 4 nM [^{3}H]-mepyramine to intact P19 EC cells, determined according to Deblasi et al. (26) in the presence of unlabelled mepyramine. In contrast to the findings with the membranes of P19 EC cells, this plot reveals two affinity binding sites. From two independent experiments, with each point in triplicate, we calculated K_d's of 4 $\pm$ 1 nM and K_d 3 $\pm$ 0.4 μM with binding site densities of 27500 $\pm$ 3500 and $15x10^6$ $\pm$ $3x10^6$ (mean $\pm$ s.e.m., n=2) sites per cell respectively. To characterize these

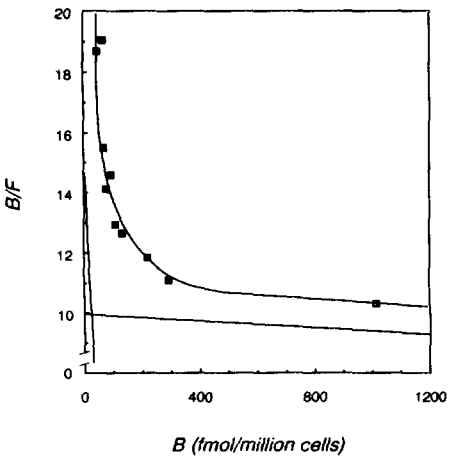

FIGURE 3. Scatchard plot of the binding of 4 nM [^{3}H]-mepyramine to intact P19 EC cells in the presence of a concentration range (1-250 nM) unlabelled mepyramine. The plot was fitted according to a two sites relation. The graph represents one out of two experiments.

two sites further we performed a displacement study with cicletanine, making use of our observation that this H_1-antagonist shows a large degree of stereospecifity on the P19 EC cell membranes. From the IC_{50} of (-)cicletanine a K_i-value of 20 nM was deduced, similar to that found on the membrane fraction of these cells. Although we found no complete displacement by (+)cicletanine, the IC_{50} for this isomer was at least a 100-fold greater than for (-)cicletanine, indicating a clear stereoselectivity of cicletanine for [^{3}H]-mepyramine binding on intact cells (results not shown). Since the displacement of [^{3}H]-mepyramine by cicletanine is monophasic and stereoselective in the same concentration range as was found on the membrane preparation, we conclude that the high affinity binding sites on the intact P19 EC cells are likely H_1-receptors. We assume that the low affinity sites on the intact cells represent intracellular trapping of the radioligand.

In conclusion, this study marks the first characterization of a receptor for histamine in cells analogous to those present in early embryonic development. Currently, we use the method of [^{3}H]-mepyramine binding to intact cells to study the H_1-receptor in other ES and EC cell lines. We apply the single cell Ca^{2+} measurements to investigate the regulation of the expression of functional histamine receptors in differentiated cells, derived from undifferentiated embryonal carcinoma and embryonic stem cells as they differentiate. Preliminary results suggest a differential regulation of the H_1-receptor as the cells differentiate. Histamine may therefore represent a new factor for processes occuring during preimplantation development of the mouse.

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