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H_1 AND H_2 HISTAMINE RECEPTORS IN *N*-NITROSO-*N*-METHYLUREA (NMU)-INDUCED CARCINOMAS WITH ATYPICAL COUPLING TO SIGNAL TRANSDUCERS

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Abstract—Two specific binding sites for histamine were characterized in the cell membrane of *N*-nitroso-*N*-methylurea (NMU)-induced tumors. The first one, with higher affinity ($K_d = 4 \pm 2$ nM), was further identified as an H_2 type, while the lower affinity one (35 ± 10 nM) corresponded to an H_1 receptor. Histamine concentrations up to 50 nM, as well as H_2 agonists, significantly enhanced the phosphoinositide turnover by acting through higher affinity H_2 receptors. On the other hand, histamine at concentrations over 50 nM and H_1 agonists produced a 100% increase in cAMP levels in a response specifically blocked by mepyramine. These H_1 and H_2 histamine receptors that exhibit different linkages to second messenger systems may prove to be a characteristic of cells with a high proliferating capacity, such as undifferentiated or transformed cells.

Key words: histamine receptors; signal transduction; NMU-induced carcinomas

The role of histamine in tumor development has been discussed extensively. A high histamine content and biosynthesis have been reported in many experimental and human neoplasias [1, 2]. The presence of histamine receptors has been demonstrated in different cell lines derived from human neoplasias [3] and in human mammary carcinomas [4].

A role for histamine as a growth factor has been reported in different tumor-derived cells [5], as well as an inhibitory effect on cell proliferation produced by H_2 antagonists in murine and human melanoma cells [6].

Working with an experimental mammary carcinoma chemically induced in rats, we have reported previously an increased histamine biosynthesis [7] and the expression of histamine receptors on the cell membrane [8, 9]. In rats, the *in vivo* treatment of such tumors with H_2 antagonists produces a significant tumor regression. When tested *in vitro*, using the clonogenic agar technique, histamine or H_2 antagonist treatment inhibited cell proliferation by 60% [9, 10].

Here we studied the pharmacological properties and the associated second messenger systems of these histamine receptors, in order to elucidate the role of histamine in the growth and development of these experimental mammary carcinomas.

MATERIALS AND METHODS

Chemicals. Histamine dihydrochloride [ring, methylene-^{3}H], [^{3}H]tiotidine, [^{3}H]mepyramine, [^{3}H]*myo*-inositol and [^{3}H]cAMP were purchased from New England Nuclear (Boston, MA, U.S.A.). Histamine dihydrochloride, mepyramine hydrochloride, famotidine, ranitidine, *myo*-inositol and cAMP were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The H_1 agonists 2-(3-fluorophenyl) histamine and 2-(3-trifluoromethylphenyl) histamine and the H_2 agonist dimaprit were provided by Dr. W. Schunack and Dr. A. Buschauer from Freie Universität, Berlin, Germany. Dowex AG-1X8 formate form was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Tumor induction. Tumors, histopathologically classified as mammary ductal carcinomas, were obtained by i.p. administration of three 50 mg/kg doses of (NMU)† to inbred Sprague–Dawley female rats aged 50, 80 and 110 days, as previously described [7, 11].

Receptor binding experiments. A purified membrane fraction was prepared as described previously [4, 8]. [^{3}H]Histamine, [^{3}H]mepyramine and [^{3}H]-tiotidine were employed as radioligands in concentrations ranging from 1 to 70 nM. [^{3}H]Tiotidine binding assays were performed in phosphate buffer medium, pH 7.4, and 1 mM histamine was employed for determining the non-specific binding. For the other radioligands, 50 mM Tris–HCl buffer was employed and a 10 μM concentration of the corresponding non-labeled ligand was used to determine the non-specific binding. Incubations were performed in triplicate, in a final volume of 300 μL during 40 min at 4° and stopped by rapid filtration through Whatman GF-B glass filters. Bound radioactivity was determined by liquid scintillation counting. The binding data were evaluated by using a non-linear curve fitting program developed in our laboratory [12].

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† Abbreviations: NMU, *N*-nitroso-*N*-methylurea; cAMP, cyclic AMP; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

For displacement studies, 10 nM [^{3}H]tiotidine or 10 nM [^{3}H]mepyramine was incubated with increasing concentrations of histamine or with different histamine agonists or antagonists (1 nM to 10 mM). Experiments were performed using the above-described conditions.

cAMP measurements. Tissue slices of approximately 50 mg were incubated for 3 min in Hanks' medium in the presence of methyl-isobutyl xanthine (1 mM) as described elsewhere [8, 13] and then stimulated for 10 min with different concentrations of histamine, or of H_1 or H_2 agonists (10 nM to 10 μM). Incubations were stopped by rapid homogenization in ice-cold ethanol and then centrifuged for 15 min at 1200 g. The supernatant was dried, and the residue was resuspended to determine the cAMP content by the competitive protein binding assay [14]. Protein concentration was assayed by the Lowry method using bovine serum albumin as standard, and results are expressed as picomoles of cAMP per milligram of protein.

H_1 and H_2 antagonists were added to a 10 μM concentration and preincubated for 1 min prior to stimulation with histamine.

Phosphatidylinositol turnover. Tissue slices of approximately 50 mg were incubated at 37° for 2 hr with [^{3}H]*myo*-inositol (2 μCi/mL), then washed carefully and further incubated with LiCl (10 mM) for 10 min. After 20 min of stimulation with histamine, or with H_1 or H_2 agonists (10 nM to 10 μM), slices were homogenized in methanol : chloroform : water (2 : 1 : 1) and centrifuged for 15 min at 700 g. Total [^{3}H]inositol phosphates present in the aqueous phase were obtained after anion-exchange chromatography as described previously [13]. The eluted fractions were transferred to vials containing scintillation solution (PPO, POPOP, toluene and Triton 30%), and radioactivity was determined by liquid scintillation counting. Protein concentration was assayed by the Lowry method using bovine serum albumin as standard, and results are expressed as disintegrations per minute per milligram of protein.

The H_1 and H_2 antagonists were added to a 10 μM concentration and preincubated for 1 min prior to stimulation with histamine.

Statistics. Results are expressed as mean ± SEM of at least three independent experiments with a few exceptions, as noted, and statistical significance was analyzed by one-way ANOVA.

RESULTS

As previously described [8, 9], two specific and saturable binding sites for [^{3}H]histamine were characterized on the plasma membrane of NMU-induced tumors. The site of higher affinity presented an equilibrium dissociation constant (K_d) of 4 ± 2 nM and a maximal binding capacity of 80 ± 22 fmol/mg protein. The lower affinity site, with a K_d = 35 ± 10 nM, showed a higher concentration of binding sites, 260 ± 65 fmol/mg protein. The specific binding of [^{3}H]tiotidine to a purified membrane fraction was found to be saturable and with relatively high affinity. Analysis of binding data by non-linear regression indicated an equilibrium dissociation constant (K_d) of 10 ± 2 nM and a maximal binding capacity (B_{max}) of 130 ± 10 fmol/mg protein (Fig. 1). Transformation of the specific binding into a Scatchard plot resulted in a linear relationship (inset Fig. 1).

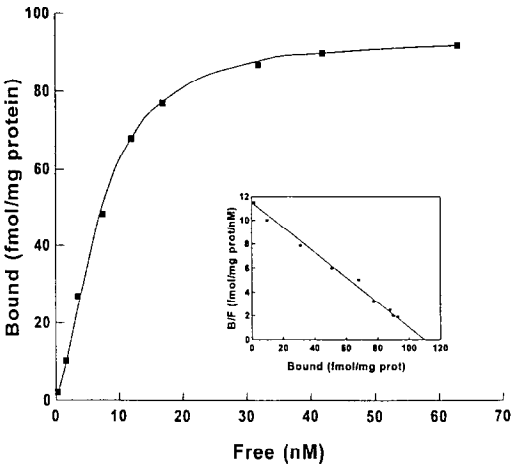

Fig. 1. Saturation experiment for [^{3}H]tiotidine binding to tumor cell membrane fraction. The curve indicates the presence of a high affinity specific binding site, K_d = 10 ± 2 nM. The inset shows the transformation of the data into a Scatchard plot. An experiment representative of eight independent assays is shown; each point is the mean of triplicate incubations.

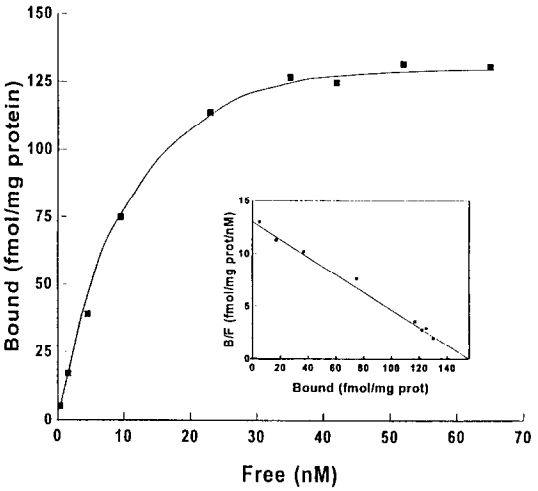

Fig. 2. Binding of [^{3}H]mepyramine to the cell membrane fraction of NMU-induced tumors. The data indicate the presence of a saturable specific binding site, K_d = 26 ± 7 nM. The inset shows the transformation of the data into a Scatchard plot. An experiment representative of eight independent assays is shown; each point is the mean of triplicate incubations.

Similarly, binding studies with [^{3}H]mepyramine indicated the presence of a lower affinity and higher

Table 1. Inhibition by various histamine agonists and antagonists of [^{3}H]tiotidine and [^{3}H]mepyramine specific binding to membranes of NMU-induced tumor cells

Compound	[^{3}H]Tiotidine		[^{3}H]Mepyramine	
	K_i (M)	IC_{50} (M)	K_i (M)	IC_{50} (M)
Tiotidine*	$3.8 \pm 1.8 \times 10^{-8}$	7.6×10^{-8}	ND†	ND
Famotidine	$1.9 \pm 0.8 \times 10^{-7}$	3.8×10^{-7}	ND	$>10^{-2}$
Ranitidine	$6.6 \pm 2.4 \times 10^{-7}$	1.3×10^{-6}	ND	$>10^{-2}$
Dimaprit	$1.1 \pm 0.6 \times 10^{-5}$	1.7×10^{-5}	ND	$>10^{-2}$
Histamine	$1.2 \pm 0.8 \times 10^{-4}$	1.8×10^{-4}	$1.5 \pm 0.4 \times 10^{-5}$	2.7×10^{-5}
Mepyramine	ND	$>10^{-2}$	$2.4 \pm 0.6 \times 10^{-8}$	4.5×10^{-8}
2-(3-Fluorophenyl) histamine	ND	$>10^{-2}$	$5.2 \pm 1.8 \times 10^{-6}$	9.6×10^{-6}
2-(3-Trifluoromethyl-phenyl) histamine*	ND	ND	$3.8 \pm 1.1 \times 10^{-6}$	6.7×10^{-6}

K_i values for the compounds were determined from their IC_{50} values according to the equation $K_i = IC_{50}/(1 + S/K_d)$, where S is the [^{3}H]tiotidine (10 nM) or [^{3}H]mepyramine (10 nM) concentration and K_d is the dissociation constant for tiotidine (10 ± 2 nM) and for [^{3}H]mepyramine (26 ± 7 nM) as determined from saturation kinetics at equilibrium. Unless noted otherwise, K_i results are means $\pm$ SD, where N = 4.

* Results are means $\pm$ range, where N = 2.

† ND, not determined.

capacity binding site on the plasma membrane, with an equilibrium dissociation constant (K_d) of 26 ± 7 nM and a B_{max} of 187 ± 23 fmol/mg protein (Fig. 2).

On cursory inspection, these data would appear to indicate that the higher affinity binding site for [^{3}H]histamine corresponded to an H_2 receptor, while the lower one presented H_1 binding properties.

Pharmacological characterization of binding sites by displacement experiments performed with [^{3}H]-tiotidine and different histamine agonists and antagonists, showed the presence of a typical H_2 binding site (Table 1). Likewise, employing [^{3}H]-mepyramine, a classic H_1 receptor was disclosed at the lower affinity site (Table 1). For both H_1 and H_2 receptors, the inhibition constant (K_i) values of histamine and competing agents were very similar to corresponding values in reference systems.

Second messenger production induced by histamine was studied in the NMU tumors. Exposure of tumor tissue to low concentrations of histamine, such as 10 nM, led to a rapid decrease in cAMP content as previously reported [8]. On the contrary, histamine concentrations over 50 nM produced a concentration-dependent increase in intracellular cAMP levels (Fig. 3). Maximal response was observed above 10 μM, and the EC_{50} value was 0.25 μM histamine (see Fig. 5). A similar concentration-dependent response was observed with the H_1 agonist (Fig. 3); the corresponding EC_{50} value was 0.25 μM 2-(3-fluorophenyl) histamine. This increase in nucleotide levels was specifically and entirely abolished by the H_1 antagonist mepyramine, whereas the H_2 antagonists cimetidine or ranitidine produced a 100% potentiation of response to 10 μM histamine (Fig. 3). These data indicated that the cAMP production was mediated by the stimulation of the lower affinity H_1 receptor.

Pertinent studies demonstrated that the H_2 receptor was linked to the enhancement of phosphoinositide production. Low histamine concentrations up to 50 nM produced a 100% increase

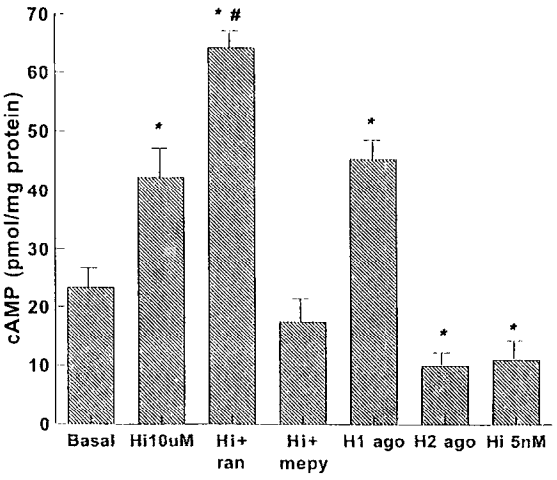

Fig. 3. Increase in cAMP levels induced by high histamine concentrations and an H_1 agonist. The histamine antagonists mepyramine (mepy) and ranitidine (ran), the H_1 agonist 2-(3-fluorophenyl) histamine (H_1 ago), and the H_2 agonist dimaprit (H_2 ago) were added at a 10 μM concentration. Data shown are the means + SEM from three independent experiments. Key: (*) significant difference compared with the basal value ($P < 0.01$), and (#) significant difference ($P < 0.01$) compared with 10 μM histamine or the H_1 agonist response (one-way ANOVA).

in phosphatidylinositol turnover with the maximal effect observed at 5 nM. This response was selectively inhibited by H_2 antagonists, while H_1 receptor antagonists, such as mepyramine, showed no effect (Fig. 4). No modification in phosphoinositide production was observed with higher histamine concentrations (reaching 100 μM). The H_2 agonist dimaprit produced a concentration-dependent increase in phosphatidylinositol hydrolysis with an

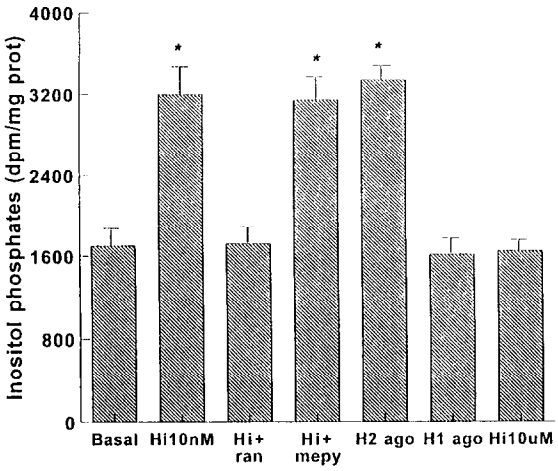

Fig. 4. Total inositol phosphate production in NMU experimental tumors induced by low histamine concentrations and an H_2 agonist. Ranitidine (ran), mepyramine (mepy), the H_1 agonist 2-(3-fluorophenyl) histamine (H_1 ago), and the H_2 agonist dimaprit (H_2 ago) were added at a 10 μM concentration. Data shown are the means ± SEM of three independent experiments. Key: (*) significant differences compared with basal conditions ($P < 0.01$, ANOVA).

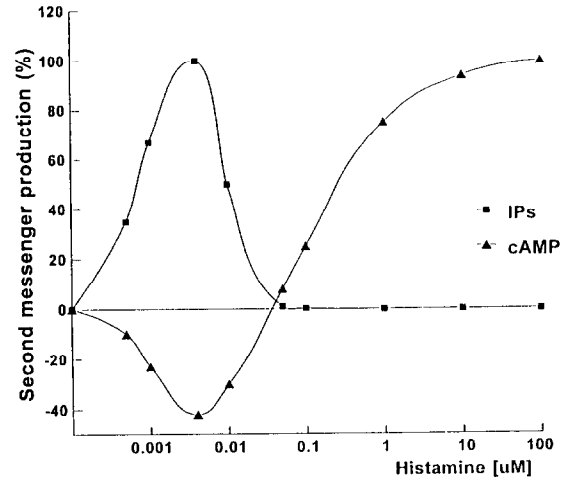

Fig. 5. Concentration–response curves showing the simultaneous effect of histamine on cAMP production and on phosphoinositide turnover.

EC_{50} value of 0.10 μM and a maximal effect at a 10 μM concentration (Fig. 4).

The action produced by different histamine concentrations on the two transductional pathways studied is represented in Fig. 5. At a low concentration range, histamine enhanced phosphatidylinositol hydrolysis, while at concentrations above 50 nM, it induced a concentration-dependent increase in cAMP levels. However, within the same low range, concentrations below 50 nM decreased cAMP levels.

The histamine concentration range able to stimulate both metabolic pathways corroborated that the binding site of higher affinity characterized with [^{3}H]histamine corresponded to an H_2 receptor and the one of lower affinity to an H_1 receptor.

DISCUSSION

Both H_1 and H_2 histamine receptors are co-localized in various cells or tissues, and regulate histamine responses via secondary Ca^{2+} and cAMP pathways, respectively [15]. H_1 receptors are generally coupled to the phosphatidylinositol hydrolysis pathway with subsequent mobilization of intracellular calcium [16, 17], whereas H_2 histamine receptors are positively coupled to adenylyl cyclase and stimulate intracellular cAMP formation [18, 19].

The present results indicate that in these experimental tumors high-concentration histamine, as well as the H_1 agonist, produces an increase in cAMP levels. The H_1 antagonist specifically blocked, while the H_2 antagonists potentiated, this effect of histamine, reinforcing the hypothesis that this response is a direct action of histamine exerted through lower affinity H_1 receptors.

Such findings are consistent with the concentration-dependent inhibition of cell proliferation induced by histamine and an H_1 agonist when tested *in vitro* using the clonogenic agar technique [9]. Identical EC_{50} values were determined for both responses: cAMP production and decrease in colony formation in NMU-induced mammary carcinomas [20]. The inhibitory effect on tumor growth induced by cAMP and different agonists has been well established [21]. In agreement, the stimulation of an H_1 receptor coupled to adenylyl cyclase produced inhibition of tumor growth. We have reported previously that *in vivo* treatment with H_1 antagonists enhances tumor growth and development [9, 10].

On the other hand, the high affinity H_2 histamine receptor characterized in the NMU experimental tumors mediated their effect via the products of inositol phospholipid hydrolysis. Phospholipase C-mediated phosphoinositide hydrolysis, with subsequent Ca^{2+} mobilization and protein kinase C activation, is a major growth regulatory signal [22]. Therefore, these H_2 histamine receptors are coupled to a proliferating biochemical pathway. Studies performed with NMU tumor cells cultured *in vitro* showed that treatment with H_2 agonists resulted in a significant increase in the number of colonies grown in soft agar, while H_2 antagonists produced a clear inhibitory effect on cell proliferation [20]. Likewise, *in vivo* treatment of rats with H_2 antagonists led to a significant inhibition on tumor growth and development [9, 10].

The above data corroborate our previous findings on a growth control mechanism regulated by histamine present in the NMU-induced mammary carcinomas [10, 20].

It has been reported recently that in HL-60 promyelocytes histamine increases cytosolic Ca^{2+} via H_2 receptors, suggesting the involvement of a different H_2 receptor subtype [23, 24].

A heterogeneity of H_1 receptors has been reported, including subclasses of H_1 receptor detected in various tissues using different radiolabeled ligands [16, 25]. At present, there are no reports concerning an H_1 receptor coupled to adenylyl cyclase activation in any tissue. An indirect stimulation of cAMP production has been described in brain slices, consisting of a large amplification of the cAMP response produced by H_2 receptors [26]. The H_1 receptor-mediated cAMP production observed in NMU experimental tumors cannot be explained by an indirect effect, as the simultaneous blockade of H_2 receptors with an antagonist led to a 100% potentiation of the H_1 response and, moreover, H_2 receptor stimulation failed to increase cAMP production.

Our findings clearly show that histamine receptors characterized in the experimental carcinomas are associated with intracellular second messenger systems distinct from the ones normally described in mammalian cells. The pharmacological properties of these receptors, as determined by the displacement experiments with different agonists and antagonists, indicate that membrane receptor proteins correspond to typical H_1 and H_2 receptors. The K_i values obtained are closely similar to corresponding values for reference biological systems [16, 19, 27, 28].

NMU produces a point mutation on the Ha-*ras* oncogene with subsequent expression of an oncogenic p21 Ras protein [29]. The Ha-*ras* oncogene and the oncogenic form of the p21 Ras protein may be involved in the different receptor linkage. Moreover, the expression of diverse G-proteins as a result of the activation of other oncogenes may also explain this change in histamine receptor coupling to transductional pathways.

Although no attempt was made here to determine the percentage of NMU-induced mammary carcinomas that express the mutated Ha-*ras* oncogene, this fraction is estimated to be very high, around 90%, as reported for Sprague–Dawley rats [27]. In this work, out of 36 different tumors studied only 3 (less than 10%) failed to exhibit this abnormal linkage to second messenger systems. Likewise, *in vivo* treatment of NMU-induced tumors in rats with H_2 antagonists indicated that around 10% of the tumors are unresponsive to this therapy [9, 10], and a similar percentage was recorded *in vitro* with H_2 antagonist treatment. In agreement, it has been demonstrated that transformation of Balb/T3T cells with the viral k-*ras* oncogene results in the expression of an altered H_1 histamine receptor signaling [30].

This atypical histamine receptor coupling may represent a characteristic of cells with a high proliferating capacity, such as undifferentiated or transformed cells. We have reported an identical histamine receptor association with signal transducers during the development of normal rat mammary glands [13]. The presence of a high affinity histamine receptor (H_2) coupled to a proliferative biochemical pathway enables low histamine concentrations to induce cell growth.

To conclude, our findings demonstrate a critical role for histamine in tumor development, and they contribute to elucidating the complex mechanism through which histamine may regulate cell proliferation in normal and in pathological tissue.

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