Molecular characterization of specific H1-receptor agonists histaprodifen and its Nα-substituted analogues on bovine aortic H1-receptors

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Molecular characterization of specific H$_1$-receptor agonists histaprodifen and its N$^\alpha$-substituted analogues on bovine aortic H$_1$-receptors

Abstract We determined the molecular properties of the selective and potent H$_1$-receptor agonist histaprodifen and its N$^\alpha$ substituted analogues: methyl-, dimethyl-, and imidazolylethyl-histaprodifen (suprahistaprodifen). All derivatives show high affinity for ^{3}H-mepyramine labeled bovine aortic H$_1$-receptor binding sites with the following order of potency: suprahistaprodifen > dimethylhistaprodifen > methylhistaprodifen > histaprodifen > histamine. Suprahistaprodifen and dimethylhistaprodifen were the most potent displacers of ^{3}H-mepyramine binding (K$_i$=4.3 and 4.9 nM, respectively). Histaprodifen, methylhistaprodifen and suprahistaprodifen binding was differentially influenced by GTP, whereas dimethylhistaprodifen was not affected. All drugs, except dimethylhistaprodifen, were activators of G-proteins. Their order of potency was suprahistaprodifen > histamine > histaprodifen > methylhistaprodifen. Their effect on G-protein activation was abolished by the addition of the H$_1$-receptor antagonist triprolidine (10 μM), which given alone did not activate G-proteins. Our data suggest that histaprodifens are potent but heterogeneous H$_1$-receptor ligands with diverse effects on the molecular level in our model system. While the histaprodifen, methylhistaprodifen and suprahistaprodifen data are in agreement with their agonistic nature, as shown in the functional studies performed on different species (rat and guinea pig H$_1$-receptor), dimethylhistaprodifen behaved as an antagonist in our study.

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Introduction

Histamine exerts diverse physiological and pathophysiological effects through mediation of the histamine H$_1$-receptor subtype (Hill 1990; Göthert et al. 1995; Leurs et al. 1995). In the cardiovascular system, histamine is a mediator of vasoconstriction via vascular smooth muscle H$_1$-receptors and of vasodilatation or an increase in permeability promoted by endothelial H$_1$-receptors (Toda 1987; Satoh and Inui 1984; Malinowska and Schlicker 1993). Marked variations in the H$_1$-receptor responses in a given regional vasculature were observed indicating differences in the functional and molecular properties of H$_1$-receptors throughout the vascular system (Čarman-Kržan et al. 1997; Čarman-Kržan 1989, 1994). In an attempt to better characterize the H$_1$-receptor subtype, new compounds with higher agonistic activity and receptor subtype selectivity have been developed (for review see Van der Goot and Timmerman 2000; Koper et al. 1990; Zingel et al. 1995). One of the more successful newer classes of histamine derivatives are halogenated 2-phenylhistamines, which have shown improved agonistic activity compared to the endogenous ligand–histamine (Dziuron and Schunack 1975; Zingel et al. 1990, 1995; Kramer et al. 1998). Of the halogenated derivatives, 2-(3-trifluoromethylphenyl)histamine has proven to be the most potent agonist in functional pharmacological studies (see Alexander et al. 2001), showing 128% relative activity in the guinea pig ileum in comparison to histamine (100%) (Leschke et al. 1995). In in vitro binding studies all meta substituted 2-phenylhistamines showed high affinities for H$_1$-receptor binding sites in the vascular membranes of bovine aorta. In that study, fluorinated derivatives (2-(3-fluorophenyl)histamine and 2-(3-trifluoromethylphenyl) histamine) were the most potent displacers of ^{3}H-mepyramine binding compared to histamine (Čarman-Kržan et al. 1997).

Better selectivity and potency for the H$_1$-receptor was further achieved by the synthesis of 2-(3,3-diphenyl-

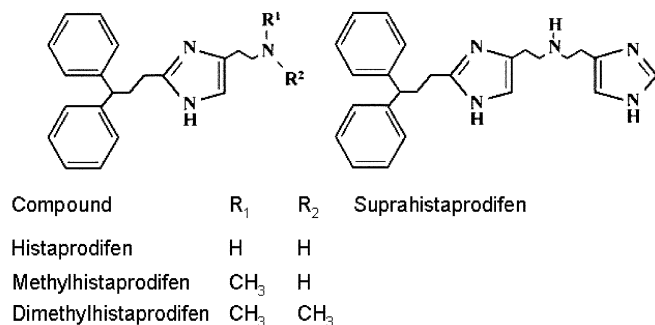| Compound	R_1	R_2	Suprahistaprodifen
Histaprodifen	H	H	
Methylhistaprodifen	CH_3	H	
Dimethylhistaprodifen	CH_3	CH_3	

Fig. 1 Structures of the four H_1-receptor ligands examined in the present study

propyl) derivatives of histamine – the histaprodifens: histaprodifen and its N^α and N^α, N^α substituted derivatives methylhistaprodifen and dimethylhistaprodifen (Elz et al. 2000a). They display full (histaprodifen) or partial agonism toward the H_1-receptor in the model system of isolated guinea pig ileum. Their effects were susceptible to blocking by the H_1-receptor antagonist mepyramine. Methylhistaprodifen was the most potent histaprodifen derivative, exceeding the potency of histamine on guinea pig ileum by a factor of 3 to 5. Its potency was further improved by the synthesis of suprahistaprodifen (N^α-(imidazolyl)ethyl)histaprodifen, the first H_1-receptor agonist with a nanomolar potency (36-fold potency compared to histamine at the guinea pig ileum histamine H_1-receptors) (Schunack et al. 2000). The histaprodifens under examination did not stimulate guinea pig atrial H_2-receptors or ileal H_3-receptors (Elz et al. 2000b; Menghin et al. 2001). Their hypotensive effect was not affected by the addition of H_2- or H_3-receptor antagonists (Schlicker et al. 2001). Their antagonist affinity in several functional adrenoreceptors and 5-HT receptor assays was low or not measurable, whereas no agonist effect was observed (Elz et al. 2000a). Further histaprodifen analogues are still under investigation (see Kramer et al. 1999; Schlicker et al. 2001; Menghin et al. 2001).

In the present study, the molecular characteristics of histaprodifen and its N^α-substituted derivatives (methyl-, dimethyl- and imidazolylethyl-histaprodifen) (for chemical structure see Fig. 1) were determined using model system of H_1-receptors in isolated bovine vascular smooth muscle membranes. We assessed the binding characteristics of these compounds for the H_1-receptor labeled with ^{3}H-mepyramine. Our assumption was that the ligand- (agonist) occupied H_1-receptor stimulates signal onset by acting as a GTP exchange factor on the G-protein (Pratt and Taylor 1990; Gutowski et al. 1991). Therefore, two approaches were used on the molecular level to differentiate between the agonistic/antagonistic nature of the ligands used: susceptibility of their affinities for the H_1-receptor to regulation by GTP in the binding studies, and their effects on G-protein activation.

Materials and methods

Animals. Bovine thoracic aortas from young male animals were freshly obtained from the slaughterhouse, packed in ice-cold Krebs-Ringer solution and immediately processed.

Drugs. ^{3}H-mepyramine (23.2 Ci/mmol) and [γ-^{32}P] guanosine 5′-triphosphate (6,000 Ci/mmol) were obtained from NEN; guanosine 5′-[γ-^{35}S]triphosphate (>1,000 Ci/mmol) from Amersham; triprolidine, histamine, mepyramine, diphenhydramine, guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), 5′-guanylylimidodiphosphate (Gpp(NH)p′), adenosine 5′-triphosphate (ATP), 5′-adenylylimido-diphosphate, ouabain, phosphocreatine, creatine phosphokinase, dithiothreitol, from Sigma; and S(+)-chlorpheniramine was from RBI. Histaprodifen (2-[2-(3,3-diphenylpropyl)-1*H*-imidazol-4-yl]ethanamine dihydrogen maleate), methylhistaprodifen (N^α-methyl-2[2-(3,3-diphenylpropyl)-1*H*-imidazol-4-yl]ethanamine dihydrogen oxalate), dimethylhistaprodifen (N^α, N^α-dimethyl-2[2-(3,3-diphenylpropyl)-1*H*-imidazol-4-yl]ethanamine dihydrogen oxalate), and suprahistaprodifen (N^α-(2-(1*H*-imidazol-4-yl) ethyl)histaprodifen-trihydrogen oxalate-0.75 H_2O) were synthesized at the Institute of Pharmacy, Free University of Berlin, Berlin, Germany (for chemical structure see Fig. 1). All other chemicals were of analytical grade and were obtained from Sigma or Merck.

Membrane preparation. Bovine thoracic aorta smooth muscle membranes were prepared according to the method described by Čarman-Kržan (1980, 1983). Aortas (12–15 g wet weight) were cleaned of fat and connective tissue, dissected and homogenized in an ultraturax tissue grinder in 20 volumes (w/v) of 0.01 M Tris-maleate, 0.33 M sucrose buffer containing 0.001 M $MgCl_2$, 0.001 M KCl, and 0.001 M dithiothreitol (pH 7.4). The homogenates were differentially centrifuged, 3,000 g for 20 min; 30,000 g for 30 min, then resuspended in 0.15 M Na$^+$/K$^+$ phosphate buffer (pH 7.4) and again centrifuged, 30,000 g for 30 min. The final 30,000 g pellets were resuspended in the same buffer and used for the binding assay and protein determination. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Saturation binding assay. 1.0 mg of vascular membrane protein was incubated for 15 min at 37°C in 0.15 M Na$^+$/K$^+$ phosphate buffer (pH 7.4) in a total volume of 0.5 ml with the indicated concentrations (1–10 nM) of ^{3}H-mepyramine (specific activity 23.2 Ci/mmol). Specific binding of ^{3}H-mepyramine represents the difference between total and non-specific binding (binding in the presence of 0.1 mM triprolidin) and represents 30–35% of total binding.

Competition binding assay. 1.0 mg of vascular membrane protein was incubated for 15 min at 37°C in 0.15 M Na$^+$/K$^+$ phosphate buffer (pH 7.4) in a total volume of 0.5 ml with ^{3}H-mepyramine (concentration 5 nM) in the presence of the indicated drug in the concentration range from 0.1 nM–0.1 mM or 1 mM (depending on the drug used). Competition binding experiments for all drugs were repeated under the same incubation conditions in the presence of GTP (0.1 mM) or its nonhydrolyzable analog Gpp(NH)p′ (0.1 mM). After incubation the membrane bound ligand was separated from the unbound ligand by vacuum filtration. The radioactivity trapped on a GF/C (Whatman) glass fiber filter was counted in 10 ml of Aquasol scintillation liquid (NEN) in a LKB Rackbeta 1214 liquid scintillation counter.

GTPase activity assay. Measurement of GTPase activity was performed radiometrically according to Cassel and Selinger (1976), using the modifications by McKenzie (1992) and Bavec et al. (1999). To 20 µl of the 3-fold diluted membranes we added an ice cold reaction cocktail containing: 1 mM of ATP, 1 mM of 5′-adenylylimido-diphosphate, 1 mM of ouabain, 10 mM of phospho-

creatine, 2.5 U/ml of creatine phosphokinase, 4 mM of dithiothre-itol, 5 mM of $MgCl_2$, 100 mM of NaCl, and trace amounts of [γ-^{32}P] GTP (to give 50,000–100,000 cpm in an aliquot) with the addition of cold GTP to yield the required total concentration of GTP of 0.5 μM. The total volume of the assay mixture was 100 μl, resulting in a final protein concentration of 80 μg/ml. Background low-affinity hydrolysis of [γ-^{32}P] GTP was assessed by incubating parallel tubes in the presence of 100 μM GTP. Blank values were determined in the assay mixture without membranes using Tris-EDTA-buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH=7.5). The GTPase reaction was started by transferring the reaction mixtures to a 25°C water bath for 10 min. Unreacted GTP was removed by addition of a 5% suspension of activated charcoal in 20 mM H_3PO4. The radioactivity of the residual radioactive phosphate was deter-mined in an LKB 1214 Rackbeta liquid scintillation counter. The basal GTPase activity of the vascular smooth muscle membrane preparation was 28.9±1 pmol/min/mg protein.

GTPγS binding assay. The rate of [^{35}S]GTPγS binding to bovine thoracic vascular smooth muscle membranes was determined as previously described by McKenzie (1992) with minor modifica-tion by Bavec et al. (1999). Briefly, the membranes (final protein concentration in the assay mixture was between 350 μg/ml and 500 μg/ml) were incubated with 5 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM NaCl, 1 μM GDP and 0.5–1 nM [^{35}S]GTPγS (approxi-mately 100,000 cpm per assay) at 25°C in Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH=7.5) for 4 min with or without the drugs. The unbound [^{35}S]GTPγS was washed out by rapid filtration of the reaction mixture through Millipore GF/C glass-fiber filters under vacuum three times with 5 ml of Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH=7.5). After extraction of the radio-active material overnight in 20 ml of Emulsifier-Safe (Packard, USA) scintillation liquid, radioactivity was determined using a LKB 1214 Rackbeta liquid scintillation counter. Blank values were determined by the same procedure using samples in which the membranes were replaced with buffer.

Calculations. The equilibrium dissociation constants (K_D) and the maximum binding capacity (B_{max}) for ^{3}H-mepyramine binding were determined by the method of Scatchard (1949) and were used to calculate the K_i (inhibition or affinity constant for the competing drug) for the ligands used. Displacement binding data were ana-lyzed using a non-linear curve fitting program (Graph Pad Prism). An F-test was used to evaluate whether the displacement of ^{3}H-mepyramine binding by the drugs under study was better fitted to a one- or two-site model. The K_i value was also calculated from the IC_{50} value (a concentration of competing ligand that displaces 50% of specific ^{3}H-mepyramine binding) using Cheng-Prusoff's equation: $K_i=IC_{50}/1 + (L/K_D)$, where L is the concentration of the free radioligand used in the assay and K_D is the dissociation con-stant of the radioligand for the receptor (Cheng and Prusoff 1973). Data are shown as mean ± SEM. Statistical analysis was per-formed using Student's *t*-test. A *P* value <0.05 was considered sta-tistically significant; *n* in the text refers to the number of experi-ments. The analysis of the GTPase activity and of the rate of GTPγS binding was performed via two-phase dose-response dia-grams. Parameters of dose-response curves (maximal effect, Hill coefficient (n_H), and EC_{50}) were obtained by non-linear least squares fitting of dose-response curves to the experimental points using the PRISM3 computer program (GraphPad Software, San Diego, CA, USA), which was also used for graphical presentation of the results.

Results

Saturation binding experiments

In the saturation binding studies the specific binding of ^{3}H-mepyramine to bovine vascular membranes was sat-urable, reversible, and of high affinity. Scatchard analysis
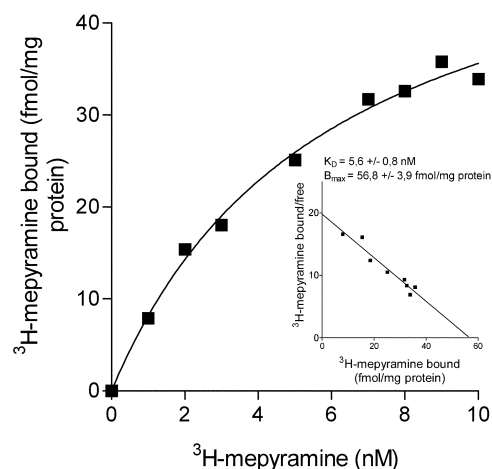

Fig. 2 Saturation isotherm of specific ^{3}H-mepyramine binding to bovine vascular smooth muscle membranes as a function of in-creasing concentration of ^{3}H-mepyramine. *Inset*: Scatchard analy-sis of the same data. The ratio of bound/free radioligand is plotted as a function of bound ligand. The slope of the line = $-1/K_D$ (r=0.98) (K_D = dissociation constant for receptor-ligand complex) and the number of binding sites (B_{max}) = intercept of the plot with the abscissa. For the ^{3}H-mepyramine binding assay see materials and methods section
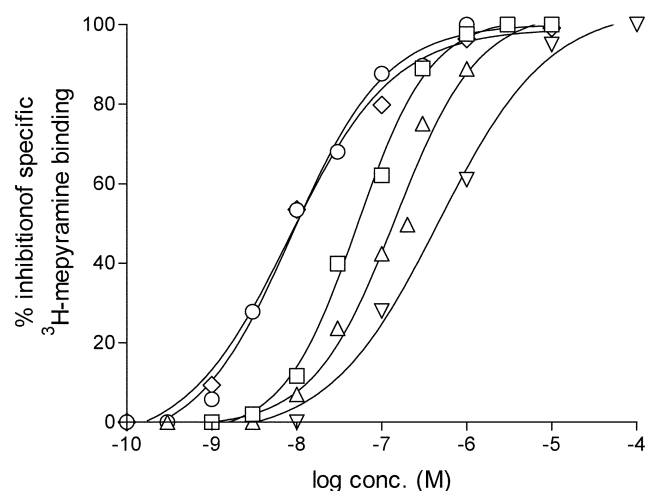

Fig. 3 Inhibition of specific ^{3}H-mepyramine binding to bovine aortic vascular smooth muscle membranes with histaprodifens: suprahistaprodifen (*diamonds*), dimethylhistaprodifen (*circles*), methylhistaprodifen (*squares*), histaprodifen (*triangles*), and hista-mine (*upside-down triangle*). Each dot represents the mean value of 3–5 independent experiments done in triplicate. The SEM value is not greater than 5%. For details see Materials and methods section

(inset) of specific ^{3}H-mepyramine binding (concentration range 1–10 nM) to bovine vascular membranes revealed a straight line, indicating the presence of a homogeneous population of ^{3}H-mepyramine binding sites with a Hill co-efficient (n_H) near unity (1.02±0.05), a dissociation con-stant (K_D) of 5.6±0.8 nM, and a maximal number of bind-ing sites (B_{max}) of 56.8±3.9 fmol/mg protein (Fig. 2).

Table 1 Effect of selective histamine H_1 receptor ligands–histaprodifen and its N^α substituted derivatives on vascular H_1 receptor binding sites and GTPase activity

Compound	Binding data			Effect on GTPase[b]		
	IC_{50} (nM)	K_i (nM)	n_H	Max effect (%)[c]	EC_{50} (nM)[d]	n_H[e]
Histaprodifen	145.0±0.09	77.6[a]	0.89±0.16	160±10	460±0.18	1.2±0.2
+ GTP	42.2±0.14	22.6[a]	0.78±0.18			
Methylhistaprodifen	50.7±0.05	27.1[a]	1.00±0.11	140±8	430±0.21	1.0±0.2
+ GTP	12.2±0.03	6.5[a]	0.84±0.05			
Dimethylhistaprodifen	9.1±0.09	4.9[a]	0.78±0.09	98.8±3	n.d.	n.d.
+ GTP	11.6±0.16	6.2[a]	0.82±0.16			
Suprahistaprodifen	8.1±0.10	4.3[a]	0.71±0.17	240±12	0.31±0.49	1.3±0.2
+ GTP	149.7±0.04	79.1[a]	1.08±0.12			
Histamine	460.0±0.15	246.0	0.67±0.15	220±15	260±0.09	0.7±0.2
+ GTP	2,320.0±0.15	1,240.0	1.06±0.29			

[a] IC_{50} and K_i values derived from displacement curves shown on Figs. 3 and 4
[b] Essentially same results were obtained also by monitoring the rate of GTPγS binding
The reported K_i values for histaprodifens and histamine indicate that the binding data were best fitted according to a one binding site model using a non-linear curve fitting

[c] Maximum GTPase activity, % of basal
[d] Concentration of half maximal activation of GTPase
[e] Hill coefficient (see Pratt and Taylor 1990)
Basal GTPase activity: 28.9±1 pmol/min/mg protein
n.d. not determined

Competition binding experiments with histaprodifens in the presence and absence of GTP

In the competition binding assay histamine H_1-receptor ligands: histaprodifen, methylhistaprodifen, dimethylhistaprodifen, suprahistaprodifen and histamine were used as displacers of specific ^{3}H-mepyramine binding to H_1-receptors in isolated vascular smooth muscle membrane from bovine thoracic aorta. Histaprodifen, its N^α methylated derivatives (methyl-, dimethyl-) histaprodifen, and suprahistaprodifen showed high affinity toward histaminergic H_1-receptor binding sites and were able to fully inhibit specific ^{3}H-mepyramine binding (concentration range 0.1 nM–0.1 mM) (Fig. 3).

The rank order of potency for the inhibition of the radioligand binding in comparison to histamine was suprahistaprodifen > dimethylhistaprodifen > methylhistaprodifen > histaprodifen > histamine. Suprahistaprodifen and dimethylhistaprodifen showed the highest affinity (K_i 4.3 and 4.9 nM, respectively) (Table 1). All histaprodifens were more potent inhibitors of specific ^{3}H-mepyramine binding than the parent drug, histamine. Histaprodifen, methylhistaprodifen, dimethylhistaprodifen, suprahistaprodifen and histamine displace ^{3}H-mepyramine monophasically (see Table 1).

Based on the observation that guanylnucleotides (GTP or its non-hydrolyzable analog Gpp(NH)p´) generally decrease the affinity of agonists for the receptor and may convert a biphasic competition binding curve to a monophasic one, they are routinely used in binding assays to differentiate between agonist- and antagonist-ligands (Pratt and Taylor 1990).

The inhibition of specific ^{3}H-mepyramine binding by various histaprodifens to the vascular H_1-receptor was

therefore performed in the presence of GTP (0.1 mM). GTP differentially affected the affinities of the tested compounds for the H_1-receptor: GTP shifted the inhibition binding curves of histaprodifen and methylhistaprodifen to the left by an order of magnitude less than 1, whereas it did not influence the inhibition curve of dimethylhistaprodifen. Suprahistaprodifen and histamine inhibition curves were shifted to the right, as expected from classical agonists (Kenakin 1993, 2002). n_H values for both drugs, although not being statistically different from unity, were relatively low in the absence of GTP and tended to be increased in its presence. In the presence of GTP, all tested compounds still remained more potent inhibitors of specific ^{3}H-mepyramine binding than histamine (Table 1).

To confirm the differences in the effect of GTP on histaprodifen, methylhistaprodifen and suprahistaprodifen binding, these experiments were repeated in the presence of non-hydrolyzable GTP analogue Gpp(NH)p´ (0.1 mM) yielding an identical result to that already obtained with GTP (data not shown).

In this and previous studies (Čarman-Kržan 1986) neither GTP nor Gpp(NH)p´ influenced the binding affinities of classical H_1-receptor antagonists (mepyramine, triprolidine, S(+)chlorpheniramine and diphenhydramine) to the vascular H_1-receptor, which is in agreement with their antagonistic nature (data not shown).

Effect of histaprodifens on GTPase activity and on the rate of GTPγS binding

Since H_1-receptors belong to the class of G-protein coupled receptors (GPCR), H_1-receptor agonists, by activat-

Fig. 4 Inhibition of specific binding of ^{3}H-mepyramine to bovine vascular smooth muscle membrane H_1-receptor by histaprodifen and its derivatives in the presence (*closed symbols*) and absence (*open symbols*) of GTP (0.1 mM). Histaprodifen (*open triangles*), histaprodifen + GTP (*closed triangles*); methylhistaprodifen (*open squares*), methylhistaprodifen + GTP (*closed squares*); dimethylhistaprodifen (*open circles*), dimethylhistaprodifen + GTP (*closed circles*); suprahistaprodifen (*open diamonds*), suprahistaprodifen + GTP (*closed diamonds*); histamine (*open upside-down triangles*), histamine + GTP (*closed upside-down triangles*); Each dot represents the mean value of 3–5 independent experiments done in triplicate. The SEM value is not greater than 5%. For details see Materials and methods section
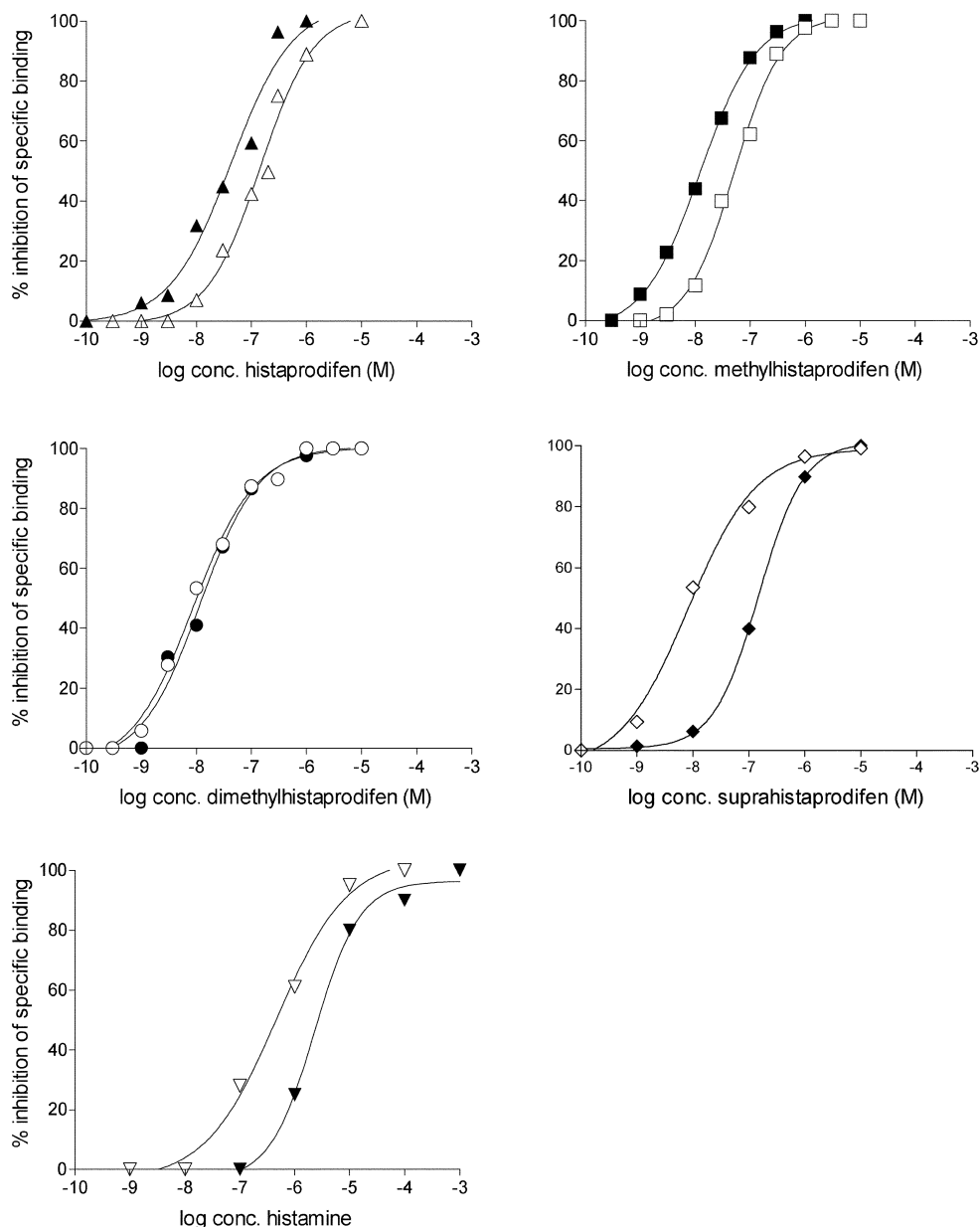
ing their receptor, activate G-proteins thereby promoting the exchange of GDP for GTP on the G_α subunit of the G-protein (Gutowski et al. 1991). We therefore tested the influence of histaprodifens on GTPase activity and GTPγS binding in the isolated vascular smooth muscle membrane and compared the results with the direct effects results of GTP on the binding of the tested compounds to H_1-receptors.

The effect of histaprodifen, methylhistaprodifen, dimethylhistaprodifen and suprahistaprodifen on the GTPase activity of the vascular smooth muscle membrane preparation is shown in Fig. 5A and Table 1. All histaprodifens including the parent compound histamine, with the exception of dimethylhistaprodifen, were able to increase basal GTPase activity (28.9±1 pmol/min/mg=100%) (Fig. 5A). The increase in GTPase activity is concentration-depen-

dent and is in accordance with a two-phase dose-response curve. Suprahistaprodifen is the most effective activator of G-proteins with the maximal effect of 240% followed by histamine (220%), histaprodifen (160%), and methylhistaprodifen (140%). EC_{50} values (nM) for the ascending phase of the dose-response curve were 460 (histaprodifen), 430 (methylhistaprodifen), 0.31 (suprahistaprodifen), and 260 (histamine). We were unable to accurately determine EC_{50} values for the descending phase since the ligand concentrations required would have been too high to determine the entire descending phase of the dose-response curve (Fig. 5A).

The effect of histaprodifens on G-proteins was additionally verified by monitoring the rate of GTPγS binding to bovine thoracic aorta membranes. We obtained essentially the same two-phase dose-response curves as shown
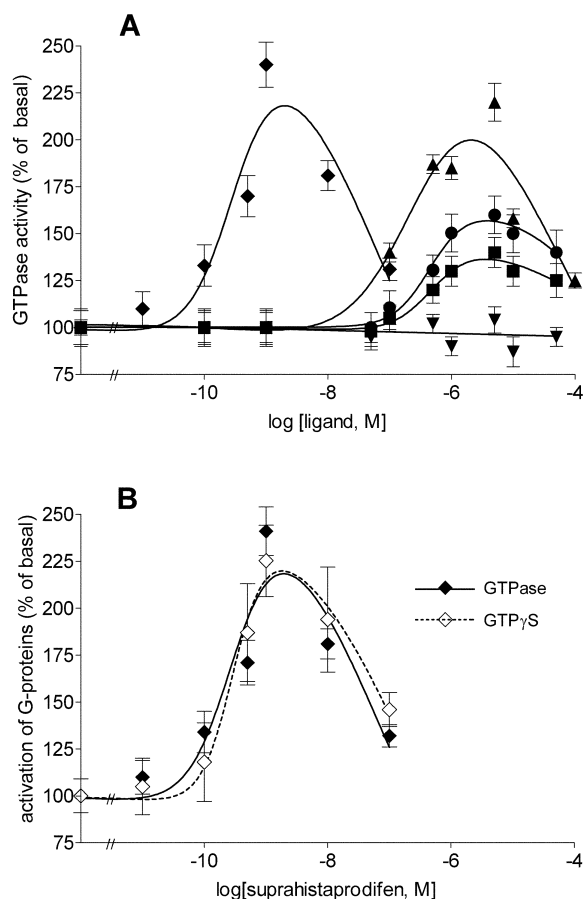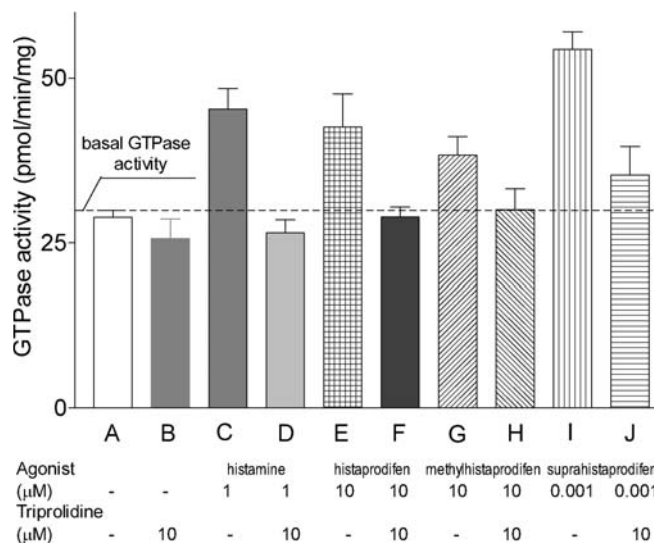
Fig. 6 Effect of triprolidine on the activation of GTPase of vascular smooth muscle membrane preparation induced by histamine, histaprodifen, methylhistaprodifen, and suprahistaprodifen. Each *bar* represents the mean value of five independent determinations with the indicated standard errors of the mean (SEM). Concentration of substrate GTP was 0.5 µM. ANOVA: F(9, 40)=47.62; at the probability level $P<0.05$ only bars *C*, *E*, *G*, and *I* were statistically significantly different from the basal (bar *A*), as revealed from Bonferroni and Tukay-Kramer multiple comparison tests

Fig. 5A, B Effect of histaprodifens on GTPase activity and on the rate of GTPγS binding to vascular smooth muscle membrane preparation. **A** Effect of histamine, histaprodifen and its derivatives on GTPase activity: histamine (*triangles*); histaprodifen (*circles*); methylhistaprodifen (*squares*); dimethylhistaprodifen (*upside-down triangles*); suprahistaprodifen (*diamonds*). Each point represents the mean value of five independent determinations; *bars* represent standard errors of the mean (SEM). Curves were obtained by fitting a biphasic dose-response equation to the corresponding experimental data using Prism 3 computer program (GraphPad, USA). Basal GTPase activity was 28.9±1 pmol/min/mg. Substrate was 0.5 µM GTP. **B** Comparison of the effects of suprahistaprodifen on GTPase activity (*closed symbols*) and on the rate of GTPγS binding (*open symbols*). Each point represents the mean value of five independent determinations of GTPase activity or the mean values of five independent determinations of the rate of GTPγS binding; *bars* represent standard errors of the mean (SEM). Curves (GTPase: *continuous curve*; GTPγS: *dashed curve*) were obtained by fitting a biphasic dose-response equation to the corresponding experimental data using Prism 3 computer program (GraphPad, USA). Basal GTPase activity was 28.9±1 pmol/min/mg. Substrate was 0.5 µM GTP. Basal rate of GTPγS binding was 37.5± 4.1 fmol/min/mg

in Fig. 5A for the GTPase activity measurement. As an example, comparison of the effects of suprahistaprodifen on G-protein activation, monitored by both methods, is shown in Fig. 5B.

The stimulation of GTPase activity induced by histamine and histaprodifens was suppressed in the presence of the H₁ antagonist triprolidine (10 µM) (Fig. 6). Triprolidine at a concentration of 10 µM was able to fully inhibit his-

tamine- (1 µM), histaprodifen- (10 µM), methylhistaprodifen- (10 µM) and suprahistaprodifen- (1 nM) induced stimulation (to the basal level) (Fig. 6). Triprolidine alone did not influence GTPase activity (Fig. 6), which is compatible with its antagonistic nature.

Discussion

Histaprodifen and its N^α substituted derivatives showed high affinity for the H₁-receptor binding site in the isolated bovine aortic membranes identified by ³H-mepyramine binding. The affinities of histaprodifen, methylhistaprodifen, dimethylhistaprodifen and suprahistaprodifen were, respectively, 3.2, 9.1, 50.2 and 57.2 fold higher than that of histamine. Comparison with the available data on human H₁-receptors binding sites expressed in COS-7 cells, reported by Bruyster et al. (2002), showed that histaprodifen and methylhistaprodifen have 20 and 40 fold higher affinities to H₁-receptor binding sites than histamine (Bakker et al. 2001), thus confirming the reported species differences in the pharmacological properties of the H₁-receptor (Yamashita et al. 1991; Mizuguchi et al. 1994).

The histaprodifens binding to H1-receptor was differentially affected by GTP (or its nonhydrolyzable analogue Gpp(NH)p´). Histaprodifen and methylhistaprodifen inhibition binding curves were shifted to the left, whereas binding of dimethylhistaprodifen was not significantly altered, the latter indicating that this drug may behave as an antagonist in our model system. Binding curves of hista-

mine and suprahistaprodifen in the presence of GTP was shifted to the right. Their inhibition binding curves tend to become steeper in the presence of GTP, which is compatible with the behavior of classical histaminergic H_1-receptor agonists (see Čarman-Kržan et al. 1997). The rightward shift in their displacement binding curves is interpreted as the result of the agonist induced formation of the complex between agonist-receptor complex and G-proteins (Kenakin 1993, 2002; Jansen et al. 1994) leading to the pharmacological effects.

The binding characteristics of histaprodifen and methylhistaprodifen in the presence of GTP (leftward shift of the competition curve) cannot be interpreted in the same way as for histamine and suprahistaprodifen. We propose that histaprodifen and methylhistaprodifen probably interact with other membrane systems that regulate G-protein signaling, e.g. the recently discovered regulators of G-proteins signaling (RGS) (Kenakin 2002; Kimple et al. 2002). However, an exact interpretation of these data requires further exploration.

The observed differences in the receptor binding characteristics of the histaprodifen derivatives under examination were compared with their ability to directly activate G-proteins. Histaprodifen, methylhistaprodifen, suprahistaprodifen and the parent histamine were able to activate G-proteins, whereas no significant activation of G-proteins was observed in the presence of dimethylhistaprodifen. As revealed from the ascending phase of the dose-response curve obtained by GTPase activity measurement and the rate of GTPγS binding, the most effective activator of G-proteins was suprahistaprodifen (maximum effect GTPase activity: 240%, EC_{50} 0.31 nM) compared to histamine (maximum effect 220%, EC_{50} 260 nM), which is in agreement with suprahistaprodifen's observed high affinity for the bovine aortic H_1-receptor (4.3 nM). The influence of GTP on suprahistaprodifen binding (shift of the inhibition curve to the right) together with its stimulation of G-proteins indicates that suprahistaprodifen acts as an agonist in our system.

Histaprodifen and methylhistaprodifen in spite of their response to the influence of GTP in our binding studies (shift of the inhibition curve to the left), were both able to activate GTPase and GTPγS binding, which is a strong indicator of their agonistic nature.

Dimethylhistaprodifen data from the binding studies (i.e., lack of GTP shift in the binding assay) and an absence of G-protein activation support our conclusion that dimethylhistaprodifen behaves as an antagonist in our model system.

H_1-receptor antagonist triprolidine at a concentration of 10 μM suppressed the stimulatory effect of histamine (1 μM), histaprodifen (10 μM), methylhistaprodifen (10 μM), and suprahistaprodifen (1 nM) on GTPase. In the control experiment triprolidine (10 μM) alone did not stimulate G-protein activation (i.e., there was no influence on basal GTPase activity and on the rate of GTPγS binding), thus-providing additional confirmation of the agonistic nature of the above drugs.

It should be mentioned that we obtained two-phase dose-response curves for G-protein activation by hista-

mine, histaprodifen, methylhistaprodifen, and suprahistaprodifen. Their EC_{50} values for the ascending phase of the dose-response curves were determined and are shown in Table 1, whereas descending phase could not be analyzed accurately. The descending part could arise from the non-specific effects of these ligands at higher concentrations (especially in the case of the first three ligands listed), from the differential effects on different types of G-proteins that might interact with the H_1-receptors, or from some other unknown mechanism.

Histaprodifens contain features characteristic of both antagonists (diphenylpropyl moiety) and agonists (histamine moiety) (Bruysters et al. 2002), in addition to different functional groups attached to the -N^α part of the molecule (-methyl, -dimethyl or -imidazolylethyl). On the basis of our results the examined histaprodifens could be divided into three distinct groups of H_1-receptor ligands:

1. Dimethylhistaprodifen (the chemical structure of dimethylhistaprodifen is closely related to the H_1-receptor antagonist diphenhydramine) showed high affinity for the vascular H_1-receptor binding sites, labeled with ^{3}H-mepyramine, which was not affected by the presence of GTP. There was also no statistically significant activation of G-proteins by dimethylhistaprodifen. Both findings led us to the conclusion that the antagonistic effects of dimethylhistaprodifen prevail in our H_1-receptor model. However, dimethylhistaprodifen was able to act as an agonist in guinea pig ileum (relative potency 242) in comparison to histamine (relative potency 100) (Elz et al. 2000a). It also showed a high potency for the H_1-receptor in the in vivo studies using a pithed and anesthetized rat model (Malinowska et al. 1999; Schlicker et al. 2001). In one of our studies dimethylhistaprodifen was also able to stimulate NGF secretion from rat neonatal cortical astrocytes in primary culture (Lipnik-Štangelj and Čarman-Kržan 2002), which indicates species differences in the pharmacological properties of H_1-receptors already shown by cloning H_1-receptors from different species (Bakker et al. 2001; Yamashita et al. 1991; Mizuguchi et al. 1994). We could also speculate that its pharmacological activity in some species/tissues is perhaps due to the activation of another intracellular system not dependent on H_1-receptor/G-protein activation (NO synthase, suggested by Malinowska et al. 1999, etc.).

2. The experiments with histaprodifen and methylhistaprodifen indicate that the classical assumption that a drug acting on a receptor requires affinity for the receptor and efficacy in producing an effect have to be interpreted in a broader sense where efficacy can be achieved by either activation of the cascade of G-proteins (one type or maybe more) through agonist binding or by activation of a broader range of molecular entities involved in the behavior of G-protein coupled receptors (interaction with the proteins that regulate receptor G-protein coupling (RGS) or mechanisms such as internalization, desensitization, oligomerization, or phosphorylation) (Kenakin 2002; Kimple et al. 2002).

The discrepancy in the histaprodifen and methyl-histaprodifen binding data (shift of the inhibition binding curves to the left in the presence of GTP), which is not characteristic of agonists, and their effects on G-proteins may be due to one of the above-mentioned mechanisms. The stimulatory effects of both histaprodifens on G-proteins suggests that they act as agonists on the bovine vascular H_1-receptor, as has already been shown for other species. Histaprodifen and methylhistaprodifen are highly potent H_1-receptor agonists (histaprodifen) or partial agonists (methylhistaprodifen) in the guinea pig ileum (relative potency 111, 343) in comparison to histamine (relative potency 100) (Elz et al. 2000a; Menghin et al. 2001). The examined histaprodifens also showed a vasodepressor response in the in vivo studies using the pithed and anaesthetized rat model (Malinowska et al. 1999; Schlicker et al. 2001). Both drugs also stimulate NGF secretion from rat neonatal cortical astrocytes through H_1-receptors (Lipnik-Štangelj and Čarman-Kržan 2002).

3. The most effective derivative of histaprodifens in our study was suprahistaprodifen, which showed the highest affinity for the bovine vascular H_1-receptor (K_i= 4.3 nM). In the presence of GTP the competition binding curve was shifted to the right, a characteristic of classical agonists such as histamine (the histamine competition curve was also shifted to the right in the presence of GTP). In addition, suprahistaprodifen was also the most effective activator of G-proteins. The nanomolar K_i value of suprahistaprodifen from our binding study correlates well with its nanomolar agonistic potency for the isolated guinea pig ileal H_1-receptor, where suprahistaprodifen was 36 times more potent than histamine, (relative potency 3,630, EC_{50} value of 5.5 nM) (Schunack et al. 2000; Menghin et al. 2001). However, suprahistaprodifen was not as effective in the in vivo systems (pithed rat), where its potency equaled that of methylhistaprodifen (Schlicker et al. 2001), thus again indicating differences in the pharmacological properties of H_1-receptors from the examined species.

In summary, histaprodifen and its analogues represent a very interesting group of potent H_1-ligands, differing in their molecular properties, in our model of the vascular H_1-receptor.

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