5j, 104619-98-9; **5k**, 104620-02-2; **5l**, 104620-05-5; **5m**, 104620-06-6; **5n**, 104619-99-0; **6a**, 65-45-2; **6b**, 17892-25-0; **7a**, 77773-92-3; **7b**, 104619-86-5; **8b**, 104619-88-7; **9a**, 104619-92-3; **9b**, 14313-44-1; **9c**, 6266-09-7; **9d**, 79230-16-3; $C_3H_7CO_2H$, 107-92-6; $C_4H_9CO_2H$, 109-52-4; $C_5H_{11}CO_2H$, 142-62-1; $C_6H_{13}CO_2H$, 111-14-8; $C_7H_{15}CO_2H$, 124-07-2; $C_8H_{17}CO_2H$, 112-05-0; $C_9H_{19}CO_2H$, 334-48-5; $H_3CCH_2COCH_2CH_3$, 96-22-0; 4-chloro-1,3-benzoxazine, 104619-87-6; 2-aminophenol, 95-55-6; 4-chloro-2-aminophenol, 95-85-2; 4-methyl-2-phenylenediamine, 496-72-0; 4-trifluoromethyl-2-phenylenediamine, 368-71-8; 4-nitro-2-phenylenediamine, 99-56-9; 2-amino-4-methylphenol, 95-84-1; 4-chloro-2-phenylenediamine, 95-83-0.

Studies on the Active Molecular Species of the H_2 Receptor Antagonists Cimetidine and Mifentidine

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The *N'*-(4-1*H*-imidazol-4-ylphenyl)formamidines were recently introduced as a new class of active H_2 antagonists; the authors of the compounds (Donetti et al. of de Angeli, Italy) have suggested that these compounds interact with the H_2 receptor through their monocations. This is at variance with the model proposed for cimetidine by the SK&F (Smith Kline & French, UK) group who proposed the neutral molecule as the species active at the H_2 receptor. In the present study we have investigated the issue whether the neutral or charged species is the active one by measuring the pA_2 values of mifentidine and cimetidine at different pH values. Changing the pH will influence the species equilibria of both compounds and thereby affect their activity. The activity changes measured for both compounds are consistent with the proposition that cimetidine as well as mifentidine elicit their activity through their neutral species.

Recently Donetti et al.[1] introduced a new class of H_2 antagonists, the *N'*-(4-1*H*-imidazol-4-ylphenyl)formamidines, of which mifentidine (Figure 1a) is a representative. It was shown that the predominant species present at physiological pH is the monocation, in which the amidino group of the *N*-isopropylformamidine is protonated and the imidazole group is uncharged. Moreover, it was suggested that (i) the charged amidino group of mifentidine upon interaction with the histamine H_2 receptor might be related to the charged imidazole of cimetidine (Figure 1b) and (ii) the imidazole ring of mifentidine might be comparable to the neutral amidine arrangement present in the cyanoguanidine group of cimetidine. That mifentidine and cimetidine occupy the same receptor site either in the classical way by assuming the same role for the imidazole groups and the amidine groups, or in the way Donetti et al. suggested might be concluded from their molecular electrostatic potentials.[2] Therefore, the speculations of Donetti et al. on the role of the imidazole moiety and the uncharged cyanoguanidine of cimetidine could lead to an interpretation on the mode of interaction of the latter molecule with the H_2 receptor different from that of Ganellin et al. The speculation of Donetti et al.[1] has important implications for studies on the interaction of histaminergic ligands at the level of the receptor and eventually for designing new active compounds. In this paper we try to answer which molecular species of the H_2 antagonists mifentidine and cimetidine interacts with the histamine H_2 receptor. The method used to get insight into this problem is based on studying the influence of pH on the binding affinity of the compounds for the H_2 receptor. This method was used previously to determine the active species of the histaminergic compounds histamine and dimaprit[3] and of two classes of β-adrenergic compounds.[4] In this paper we apply the above-mentioned technique to cimetidine and mifentidine.

Results and Discussion

The development of H_2 antagonists was based on the notion that an antagonist on the one hand should have sufficient chemical resemblance to histamine in order to be recognized by the H_2 receptor and on the other hand should be sufficiently different as well to ensure its lack of intrinsic activity. One of the first successful compounds in this respect was the partial agonist N^{α}-guanylhistamine.[5] It was discovered that elongation of the side chain influences antagonistic activity positively as for example in compound SK&F 91486,[6] in which lengthening of the guanidine side chain from an *N*-ethyl group (N^{α}-guanylhistamine) to an *N*-propyl group enhances activity. A further increase in antagonistic activity was achieved both by reducing the basicity of the guanidine group and by changing the electronic influences on the imidazole ring in order to increase the relative amount of the N^{τ}-H tautomer with respect to the N^{π}-H tautomer.[7] A favorable electronic effect could be accomplished by introducing a methyl group at position 4 in the imidazole ring and changing the side chain into *N'*-methyl-*N*-[(ethylthio)methyl]-*N''*-cyanoguanidine.[8] The electron-donating character of the methyl group at position 4 and the electron-withdrawing character of the sulfur atom in the side chain increase the relative amount of N^{τ}-H tautomer of the imidazole ring. Reduction of the basicity of the guanidine group was achieved by introduction of the strongly electron withdrawing cyano group, which reduces the pK_a of the guanidine to a value of −0.4. The conclusion

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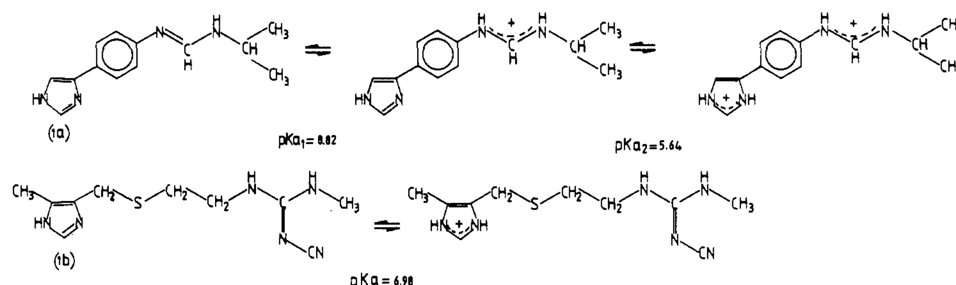

Figure 1. Ionic equilibria of mifentidine (a) and cimetidine (b) in aqueous solution.

Table I. Mole Percentage of Species at pH 7.0, 7.4, and 7.8 of Histamine, Cimetidine and Mifentidine and Their pK_a Values

	pH										
	7.0			7.4			7.8				
	ns[a]	mc[b]	dc[c]	ns	mc	dc	ns	mc	dc	pK_{a1} (±SEM)[d]	pK_{a2} (±SEM)
histamine	0.24	88.49	11.27	0.64	94.57	4.80	1.63	96.43	1.63	9.57 (0.006)	6.10 (0.01)
cimetidine	51.21	48.79		72.50	27.50		86.88	13.12		6.98 (0.08)	
mifentidine	1.43	94.40	4.10	3.60	94.73	1.66	8.66	90.70	0.63	8.82 (0.04)	5.64 (0.02)

[a] ns = neutral species. [b] mc = monocation. [c] dc = dication. [d] SEM = standard error of mean.

Table II. Determination of the Charge of Receptor Sensitivity at Different pH Values

	agonist act.[a] at pH						ratio act.[b]		ratio active species[c]		receptor sensitivity[d]	
	7.0		7.4		7.8							
	n	pD_2 (±SEM)	n	pD_2 (±SEM)	n	pD_2 (±SEM)	7.4/7.0	7.8/7.4	7.4/7.0	7.8/7.4	7.4/7.0	7.8/7.4
histamine	17	5.81 (0.05)[e]	13	5.88 (0.05)[e]	13	5.89 (±0.05)[e]	1.00	1.00	1.07	1.02	1.00	1.00

[a] See the Experimental Section for Pharmacological Methodology. [b] Ratio activity = antilog (pD_2 at pH − pD_2 at pH). [c] Ratio species = relative amount of species at pH/relative amount of species at pH. [d] The receptor sensitivity = ratio activity/ratio active species. [e] These values are not significantly different, $P > 0.05$.

Table III. Activities and the Change in Activity of Cimetidine and Mifetidine at Different pH Values

	antagonist act.[a] at pH									ratio act.[b]	
	7.0			7.4			7.8				
	n[b]	pA_2 (±SEM)	slope (±SEM)	n	pA_2 (±SEM)	slope (±SEM)	n	pA_2 (±SEM)	slope (±SEM)	7.4/7.0	7.8/7.4
cimetidine	9	6.02 (0.06)	1.01 (0.03)	5	6.40 (0.06)[c]	0.90 (0.03)	4	6.43 (0.06)[c]	1.06 (0.03)	2.40	1.00
mifentidine	4	7.34 (0.08)	1.11 (0.03)	4	7.76 (0.06)	1.16 (0.03)	4	7.93 (0.02)	1.29 (0.05)	2.63	1.50

[a] See Table II. [b] Number of determinations. [c] These values are not significantly different, $P > 0.05$.

was drawn that a neutral amidine part at a distance of 4–5 carbon atoms from the imidazole ring or a group with comparable properties provides an optimal H_2 antagonist. Other examples can be found in the molecular structures of tiotidine[9] and ranitidine.[10] A striking example that a neutral amidine part is required for antagonistic activity is given by impromidine[11] and its cyanoguanidine analogue.[12] Impromidine is a selective H_2 agonist that is 48 times more potent than histamine at the guinea pig atrium. Changing its guanidine group into a cyanoguanidine affords a compound with reduced receptor affinity and no agonistic activity. A pA_2 value of 6.11 was measured at the guinea pig atrium.

In a recent series of N'-(4-1*H*-imidazol-4-ylphenyl)-formamidines,[1] the *N*-cyano-substituted analogue, which has an uncharged amidino group, appeared to be completely inactive. On the basis of this observation, the authors speculated that the formamidine moiety of these compounds should be charged. Applying variations in the molecular structure within this series made the authors suggest that the cationic side chain of mifentidine might be comparable with the charged imidazole group of cimetidine and the imidazole ring of mifentidine with the neutral amidine moiety of the cyanoguanidine of cimetidine.

In the present study we determined the H_2 antagonistic activity of cimetidine and mifentidine on the guinea pig right atrium at different pH values. At the various pH values the relative concentrations of the species will differ so if there is a change in the amount of active species this will give a change in the pA_2 values. The obtained H_2 antagonistic activities give information on the identity of the active species. A similar approach has been used by Sterk et al.[3] for the determination of the active species of histamine and dimaprit. They showed that changes in the H_2 receptor sensitivity caused by changes in the pH are completely accounted for by changes in the active molecular species.

Tables I and II show that neither the pD_2 values for histamine nor the relative amounts of the monocation, which is thought to be the active species,[13,14] are influenced

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Table IV. Ratio in Species of Cimetidine and Mifetidine

	pH					
	7.4/7.0[d]			7.8/7.4[d]		
	ns[a]	mc[b]	dc[c]	ms	mc	dc
cimetidine	1.42	0.56		1.20	0.48	
mifentidine	2.52	1.00	0.40	2.46	0.96	0.40

[a–c] See Table I. [d] See c Table II.

by changes in pH. The fact that the activity of histamine does not change indicates that the H_2 receptor does not contain an amino acid residue titrating in the pH range 7.0–7.8 being involved in the binding process of histamine to the receptor protein molecule. Deprotonation of an amino acid residue (e.g., histidine or a carboxylate with a high pK_a) involved in binding of the agonist would affect the activity as can be seen in earlier work at the H_1 receptor.[15,16] In the latter study the effect of histamine on the guinea pig ileum has been studied in the pH range 7.1–8.3. It was shown that there was a significant change in activity in this pH range from which it was concluded that the protonation state of the receptor influences the binding process.

From the fact of the insensitivity of the histamine binding site we may reasonably assume that also the pA_2 values obtained for cimetidine and mifentidine do not need to be corrected for pH influences at the receptor.

According to the species distribution of cimetidine (Table I) at pH 7.0, 7.4, and 7.8, respectively, one anticipates a decrease in activity if the monocation is involved. However, the pA_2 values of cimetidine at different pH values (Table IV) seem to support the current idea of the SK&F investigators that the neutral species is involved in receptor interaction. When the pA_2 values at pH 7.0 and 7.4, respectively, are compared, an increase in activity is observed accompanied by an increase in the relative amount of neutral species (Tables III and IV). In these tables the results are presented as activity and species ratios. The activity ratio is defined as the quotient of the association constants of the antagonist at two different pH values. E.g., the ratio in activity from pH 7.0 and 7.4 is the quotient of (the association constant at pH 7.4)/(the association constant at pH 7.0). The species ratio is defined in a similar way to be the quotient of the amount of species at the two pH values.

The increase in activity for cimetidine (Tables III and IV) comparing the pA_2 values at pH 7.4 and 7.8 was not significant. This can be explained by the SEM of the measured pA_2 values. The average SEM is 0.06, which means that a species ratio must be larger than 1.32 or smaller than 0.76 to be significant. As can be seen from Table IV the only species that does not satisfy this condition is the neutral species of cimetidine over the range pH 7.4–7.8.

For mifentidine one expects identical pA_2 values if the active species is the monocation because its relative amount does not change over the pH range studied. However, if the uncharged species is the active one, an increase in activity at rising pH values should be observed (Tables I and IV). The data (Table III) clearly show an enhancement in activity for mifentidine at rising pH. All pA_2 values are significantly different ($P < 0.05$). All changes in activity can thus be explained by changes in concentrations of the neutral species. Though this is in good agreement with our assumption that the receptor sensitivity does not alter with pH, our approach involving examination of antagonists of both the cimetidine and mifentidine type that are neutral over the pH range studied would provide complementary insight with this matter. However, such approaches cause their own problems: e.g., mifentidine substituted with an electron-withdrawing group (CN) at the formamidino moiety is completely inactive;[1] substituents at the 5-position of the imidazole moiety of cimetidine change more than only the pK_a value.[17]

This study, however, seems to support the hypothesis that cimetidine acts through its neutral species and that mifentidine also behaves as a classical H_2 antagonist in the way that it interacts with the H_2 receptor through its neutral form.

Pharmacological Assays

Guinea Pig Atrium. Guinea pigs of male sex weighing 350–400 g were killed by a sharp blow on the head. The heart was removed quickly, and the spontaneously beating right atrium was dissected and placed in an organ bath containing 20 mL of buffer solution, gassed with oxygen containing 5% CO_2 at 37 °C. For experiments at pH 7.4 the organ bath contained a Krebs solution: 120 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2·2H_2O$, 1 mM $MgSO_4·7H_2O$, 1 mM $NaH_2PO_4·H_2O$, 25 mM $NaHCO_3$, and 6 mM glucose. At pH 7.0 the solution consisted of 128 mM NaCl, 5 mM KCl, 2.25 mM $CaCl_2·2H_2O$, 1 mM $MgSO_4·7H_2O$, 1 mM $Na_2HPO_4·12H_2O$, 8 mM $NaHCO_3$, and 6 mM glucose, whereas at pH 7.8 91 mM NaCl, 5 mM KCl, 2.25 mM $CaCl_2·2H_2O$, 1 mM $MgSO_4·7H_2O$, 1 mM $Na_2HPO_4·12H_2O$, 49 mM $NaHCO_3$, and 6 mM glucose were used.

The contractions were registered isometrically with a preload of 0.5 g. After a 30-min equilibration period in which every 10 min the buffer was refreshed, a cumulative dose–response curve with histamine was made by increasing each dose by a factor of 3 until a frequency plateau was reached. After a washing period of 30 min (three times 10 min), a second cumulative histamine curve was recorded and was used as the control curve. After a washing period as described above, three more dose–response curves were made with rising concentrations of antagonists. For cimetidine the concentrations used were 1×10^{-6}, 3×10^{-6}, and 1×10^{-5} M. For mifentidine the concentrations used at pH 7.0 and 7.4 were 3×10^{-8}, 1×10^{-7}, and 3×10^{-7} M. At pH 7.8 the following concentrations were used: 1×10^{-8}, 3×10^{-8} and 1×10^{-7} M. Between each dose–response curve there was a washing period during which the chronotropy returned to control values. There was no flattening of the curves, and the Hill coefficients did not differ significantly from unity.

The equilibration period with cimetidine was 10 min and with mifentidine 30 min before administering histamine. This was sufficient for both compounds because experiments showed that these were the shortest possible equilibration periods in which they could elicit their maximum antagonistic effect.

A nonlinear fit using the ALLFIT program[18] was used for estimating EC_{50} values. pA_2 values were calculated by means of an Arunlakshana–Schild plot.[19]

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Statistical significance of the results was determined by the Mann–Whitney U-test.[20,21] This is a nonparametric test because the null hypothesis is not concerned with specific parameters but only with the distribution of the variates. *P* values <0.05 were considered to indicate significant differences.

Determination of the Macroscopic pK_a Values. A glass electrode connected to a Philips PW 94149 ion activity meter was calibrated with buffers of pH 7.00 and 3.00. The ionic strength of the solution was 0.1635 (identical with the average ionic strength of the buffers used in the pharmacological experiments). Solutions of 2 mM mifentidine dihydrochloride, cimetidine dihydrochloride, and histamine dihydrochloride were titrated potentiometrically with 0.1 M KOH containing 63.5 mM NaCl, at 37.00 ± 0.05 °C, under N_2, on a Mettler DV 10 micropipettor. Data (pH values and amounts of added KOH; about 40/titration) were analyzed with the SCOGS (stability constants of generated species) computer program,[22-24] which was modified as follows: use of the Marquardt algorithm for minimalization;[25] iteration until optimium is obtained; calculation including 95% confidence intervals. The SCOGS computer program was used to obtain acid association constants making allowance for temperature, activity coefficients, volume of the solution, normality of the titrant, and added acid or base, following a nonlinear least-squares minimization of the added volumes of alkali. All titrations were in duplicate.

For calculation of the species distribution of the three compounds at the pH values used in the pharmacological experiments the computer program COMICS[26] (concentrations of metal ions and complexing species) was applied. This program contains a method for calculating equilibrium concentrations of all species in multimetal–multiligand mixtures from the pH of the solution, the total concentration of each metal and each complexing agent, and the relevant equilibrium constants (pK_a values and stability constants).

Chemicals. Cimetidine was supplied by SK&F (U.K.). Mifentidine was a gift from Instituto de Angeli (Italy). Histamine dihydrochloride was derived from Janssen Pharmaceutica (Belgium). All other chemicals were of analytical grade.

Registry No. Cimetidine, 51481-61-9; mifentidine, 83184-43-4.

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Xanthine Functionalized Congeners as Potent Ligands at A_2-Adenosine Receptors[†,‡]

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Amide derivatives of a carboxylic acid congener of 1,3-dialkylxanthine, having a 4-[(carboxymethyl)oxy]phenyl substituent at the 8-position, have been synthesized in order to identify potent antagonists at A_2-adenosine receptors stimulatory to adenylate cyclase in platelets. Distal structural features of amide-linked chains and the size of the 1,3-dialkyl groups have been varied. 1,3-Diethyl groups, more than 1,3-dimethyl or 1,3-dipropyl groups, favor A_2 potency, even in the presence of extended chains attached at the 8-(*p*-substituted-phenyl) position. Polar groups, such as amines, on the chain simultaneously enhance water solubility and A_2 potency. Among the most potent A_2 ligands are an amine congener, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-diethylxanthine, and its D-lysyl conjugate, which have K_B values of 21 and 23 nM, respectively, for the antagonism of *N*-ethyladenosine-5′-uronamide-stimulated adenylate cyclase activity in human platelet membranes. Strategies for the selection and tritiation of new radioligands for use in competitive binding assays at A_2-adenosine receptors have been considered.

The synthesis of potent and/or selective analogues of caffeine and theophylline as antagonists at extracellular adenosine receptors remains a challenge. Two adenosine receptor subtypes have been delineated on the basis of inhibition (A_1) or stimulation (A_2) of adenylate cyclase by adenosine and adenosine analogues. Numerous physiological functions have been shown to be regulated by either or both A_1- and A_2-adenosine receptors.[1] In many organs and cell lines, a single-receptor subtype has been found to occur. For example, in platelets and PC12 pheochromocytoma cells the existing adenosine receptors appear to be all of the A_2 subtype.[2] Fat cells are considered to contain only the A_1-receptor subtype.

Characterization of adenosine receptors with reversibly-binding radioligands has been carried out mainly with brain membranes, due to the high density of A_1 receptors in that organ. A number of radioligands for A_1 receptors have been reported. Tritiated N^6-cycloalkyladenosines[3,4] and tritiated (*R*)-N^6-(phenylisopropyl)adenosine[5] (PIA)

[†] Dedicated to Prof. B. Witkop on the occasion of his 70th birthday.

[‡] Abbreviations: DPX, 1,3-diethyl-8-phenylxanthine; NECA, *N*-ethyladenosine-5′-uronamide; XAC, xanthine amine congener (compound **6c**); HOBt, 1-hydroxybenzotriazole; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; PIA, N^6-(phenylisopropyl)adenosine.

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