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Protein-Linked Receptors Labeled by [^{3}H]Histamine in Guinea Pig Cerebral Cortex. I. Pharmacological Characterization

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SUMMARY

Binding of histamine to washed membranes from guinea pig cerebral cortex can be described empirically as two classes of distinct and independent sites (log $I_{P_1} = -8.45 \pm 0.02$, $R_{1,t} = 98 \pm 6$ pmol/g of protein; log $K_{P_2} = -6.34 \pm 0.22$, $R_{2,t} = 990 \pm 60$ pmol/g of protein). At 1.4 nM [^{3}H]histamine, the kinetics of association and dissociation are biexponential. The values of k_{-P_i}/k_{+P_i} calculated for parallel one-step processes agree well with the corresponding values of K_{P_i}. Both k_{-P_1} and k_{-P_2} are increased by 0.1 mM guanylylimidodiphosphate; apparent capacity at equilibrium is reduced for both classes of sites, with little or no change in K_{P_1} or K_{P_2}. Twenty-six H_2 and H_3 agonists and antagonists block access of [^{3}H]histamine to the same sites, and the binding patterns reveal either one or two hyperbolic terms [i.e., $\Sigma_{i=1}^{n} F'_i/(K_i + [L])$]. Two terms are required for six agonists and six antagonists, and F'_2 varies widely from ligand to ligand. Also, the quantity log (K_2/K_1) is correlated with F'_1 among agonists but with F'_2 among antagonists ($K_1 < K_2$). The pharmacological selectivity is suggestive of both H_2 and H_3 receptors. An H_2 specificity emerges from the appropriate values of K_i for 12 H_2 agonists (i.e., K_1 when $n = 1$ and K_2 when $n = 2$; $p = 0.00045$), although a specificity distinct from that of H_2 receptors is found with H_2 antagonists. An H_3 specificity emerges from the inhibitory potencies (IC_{50}) of eight H_3 agonists ($p = 0.00025$) and eight H_3 antagonists ($p = 0.0019$); also, the sites labeled by [^{3}H]histamine resemble H_3 receptors reportedly labeled by N^{α}-[^{3}H]methylhistamine and (*R*)-α-[^{3}H]methylhistamine. Ligand-dependent differences in F'_2 are inconsistent with the notion of distinct and independent sites, and the tendency of antagonists to promote the sites of weaker affinity (F'_2) argues against a ligand-regulated equilibrium between two states. The physical significance of the binding parameters is therefore unclear. The failure to identify an unambiguous pharmacological specificity may reflect the failure to assess binding in the correct mechanistic context.

Tritiated histamine has proven to be an enigmatic probe (1, 2). Early reports on membranes from rat cerebral cortex described a homogeneous population of sites with an apparent dissociation constant of 4–9 nM and a capacity of about 100 pmol/g of protein (1, 3, 4). The pharmacological specificity was distinct from that of H_1 receptors and ambiguous at best with respect to H_2 (3, 4), but guanyl nucleotides were found to inhibit binding noncompetitively, in a manner indicative of a G protein (3). The labeled sites appeared to be distinct from the metabolic enzyme histamine-*N*-methyltransferase and unrelated to systems involved in the storage or transport of histamine (3). In later studies with highly washed membranes, Steinberg *et al.* (5) identified two classes of sites for [^{3}H]histamine ($K_{P_1} = 3.9$ nM, $K_{P_2} = 221$ nM). Those of higher affinity closely resembled the sites described previously (1, 3, 4), not only in their affinity and capacity for the radioligand but also in the kinetics of binding and in their sensitivity to guanyl nucleotides. Inhibition of radioligand binding by 14 H_2 antagonists revealed a pharmacological specificity indicative of H_2 receptors, in contrast to the uncertainty associated with earlier results, but inhibitory potency failed to reflect the concentration of [^{3}H]histamine and other ligands in a strictly competitive fashion (6). H_2 agonists were found to inhibit binding in a characteristic and manifestly biphasic manner, and some aspects of the binding were suggestive of H_2 receptors; as found with antagonists, the inhibitory behavior of agonists was not strictly competitive (7).

The sites labeled by [^{3}H]histamine seem to be distinct from those labeled by H_2 antagonists. Saturable binding of [^{3}H]tiotidine and [^{125}I]iodoaminopotentidine has been demonstrated in membranes from various tissues, including guinea pig cortex, and the labeled sites bind H_2 antagonists with a pharmacological specificity characteristic of H_2 receptors (8–11). The pattern therefore differs from that found in earlier studies on the binding of [^{3}H]histamine (3, 4) but resembles that described by Steinberg *et al.* (6). Among the relatively few H_2 agonists examined with two or more radioligands, there is no general relationship between affinities inferred from the inhibition of either [^{3}H]tiotidine (8, 10) or [^{125}I]iodoaminopo-

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; GMP-PNP, guanylylimidodiphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid.

tentidine (11) binding on the one hand and [^{3}H]histamine binding on the other (3, 7). The effects of guanyl nucleotides on the binding of agonists are small with [^{3}H]tiotidine (10) or [^{125}I]iodoaminopotentidine (11); also, the Hill coefficients in the absence of nucleotide are typically near 1, in contrast to the lower values characteristic of G protein-linked receptors. Guanyl nucleotides reduce the specific binding of [^{3}H]histamine by 35–95%, depending on the conditions. Specific binding of [^{3}H]tiotidine has not been demonstrated in cortical membranes from rat. The H_2 gene has been detected on Northern blots (12), however, and the capacity for the more potent antagonist [^{125}I]iodoaminopotentidine is about 10 pmol/g of protein (11). In contrast, the capacity for high affinity binding of [^{3}H] histamine is about 100 pmol/g of protein (3, 6).

Genes coding for what appear to be species homologues of an H_2 receptor have been obtained from canine (13), rat (12), and human (14) genomic libraries. Cells transfected with the canine and human genes exhibited a dose-dependent increase in cellular cAMP upon the exogenous administration of histamine, and the response to 1 μM histamine was blocked by 10–100 μM cimetidine; the cells also bound [^{3}H]tiotidine, and binding was inhibited by cimetidine with a potency of 50–550 μM (13, 14). The encoded proteins exhibit a high degree of homology with other G protein-linked receptors, particularly in those regions that comprise the seven hydrophobic domains. Northern blots have failed to detect the canine gene in heart, ileum, or liver (13) or the rat gene in heart, lung, kidney, or ileum (12); similarly, specific binding of [^{3}H]tiotidine appears to be absent from peripheral tissues such as guinea pig gastric mucosa, guinea pig right atrium, and rat uterus (8), all of which are known to contain H_2 receptors.

Sites labeled by [^{3}H]histamine in various tissues closely resemble those labeled by N^α-[^{3}H]methylhistamine (15–17) and (R)-α-[^{3}H]methylhistamine (18) in cortical membranes from guinea pig (16), cortical slices and membranes from rat (15, 18), and membranes from AtT-20 cells (17). (R)-α-[^{3}H]Methylhistamine and N^α-[^{3}H]methylhistamine reportedly are specific for histaminergic autoreceptors that regulate the release of histamine from presynaptic stores (16, 18); the regulatory sites exhibit a pharmacology that is neither H_1 nor H_2 and therefore have been designated H_3 receptors (1, 19–21). GMP-PNP has been found to reduce the specific binding of (R)-α-[^{3}H]methylhistamine in cortical membranes from rat (18) and of N^α-[^{3}H]methylhistamine in sections from rat forebrain (15).

The present report describes a pharmacological characterization of the sites labeled by [^{3}H]histamine in highly washed membranes from guinea pig cortex. Elements of both H_2 and H_3 pharmacology emerge from the data, and the sites cannot be identified categorically as one receptor or the other. The ambiguity appears to derive in part from uncertainty over the mechanistic events that underlie the multiple states of affinity observed with many of the ligands tested, including antagonists. Results presented in the following paper (22) demonstrate that the apparent heterogeneity cannot be rationalized solely in terms of distinct and independent sites, and the appropriate correlate of pharmacological specificity is therefore in doubt. Preliminary reports of this work have appeared elsewhere (23, 24).

Materials and Methods

Histaminic drugs and other chemicals. [^{3}H]Histamine was obtained either from Amersham Corporation (33–54 Ci/mmol) or from New England Nuclear (32.1–48.1 Ci/mmol). Unlabeled histamine was obtained from Sigma. α,N^α-Dimethylhistamine, N^α-methyl-α-chloromethylhistamine, (R)-α-methylhistamine, and sopromidine were synthesized as described elsewhere (25–28). Ranitidine was kindly donated by Glaxo Canada Ltd. (Toronto, Canada) and tiotidine by ICI Americas Inc. (Wilmington, DE). All other histaminic drugs were the generous gift of Smith Kline and French Research Ltd. (Welwyn Garden City, UK). GMP-PNP and HEPES were purchased from Boehringer-Mannheim, and EDTA was obtained as the free acid from British Drug Houses. All buffer solutions were adjusted to pH 7.48 with NaOH. Other chemicals were reagent grade or better.

Preparation of tissue and binding assays. Cerebral cortices were obtained from male Hartley guinea pigs weighing 300–360 g. P_2 pellets were prepared as reported by Steinberg *et al.* (5) and were washed extensively in a buffer containing 50 mM HEPES, 100 mM NaCl, and 1.0 mM EDTA. Binding assays were performed according to procedures modified from those described previously (5). Washed pellets were resuspended in a buffer containing 50 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.48. The time dependence of binding was measured as described in the legend to Fig. 1 and accompanying text. The concentration dependence of binding at equilibrium was measured after incubation of the reaction mixture for 4 hr at 30°. For each assay, an aliquot of membranes (480 μl) was added to a solution of histaminic ligands and nucleotides (20 μl); the final concentration of protein generally was 0.6–1.5 mg/ml. Most assays were performed with 1.2–1.4 nM [^{3}H]histamine to permit direct comparisons of values of F_2 and F'_2 from the equations described below.

Bound [^{3}H]histamine was separated by microcentrifugation in kinetic studies and in early experiments on the concentration dependence of binding at equilibrium (5). In later experiments on binding at equilibrium, separation was achieved by rapid filtration through Whatman GF/B filters mounted on a 48-well Brandel harvester. After initial passage of the sample, the reaction tubes and the filters were washed twice with 3 ml of an ice-cold solution of NaCl (150 mM). Filters were presoaked in 0.05% polyethyleneimine and dried before use, to minimize nonspecific binding of the radioligand.

The two methods of separation were compared in five experiments on the binding of 1.4nM [^{3}H]histamine at graded concentrations of unlabeled ligands. Two times the usual number of replicated samples were prepared with aliquots of tissue drawn from the same homogenate. After equilibration in the usual manner, each set of samples was divided in two and processed one way or the other. Nonspecific binding was 2–4-fold lower when measured by filtration rather than by centrifugation; specific binding was identical, and the data were superimposable when absolute levels of binding (dpm/ml) were plotted as a function of the concentration of the unlabeled ligand.

Experiments involving graded concentrations of the radioligand typically included assays with and without GMP-PNP; preliminary studies indicated that the nucleotide is without effect on nonspecific binding, which therefore was measured only in its absence. Nonspecific binding was defined throughout as total binding in the presence of 1.0 mM unlabeled histamine. All experiments involving graded concentrations of an unlabeled ligand included controls for the binding of [^{3}H] histamine when added alone; the results were included in subsequent analyses of the data, with the concentration of the unlabeled ligand taken as 10^{-15} M (i.e., log H [A] = −15). Binding also was measured routinely in the presence of 1.0 mM unlabeled histamine; those results were omitted from the analyses except when histamine itself was the unlabeled ligand used to define the binding profile.

Radioactivity was measured by liquid scintillation counting, and rates of disintegration were determined by means of an external standard. Pellets from microcentrifugation were processed as described previously (5); filters were immersed in 3 ml of CytoScint ES (ICN Biochemicals) and vortexed several times over a period of several hours before counting. All assays were carried out in quintuplicate, and each sample was counted twice for 5 min; the 10 values were averaged to obtain the means and standard errors used in subsequent analyses.

Standard errors generally were <1% of the mean. Protein was determined by the method of Lowry *et al.* (29), with bovine serum albumin as the standard.

Analysis of data. All analyses were performed with total binding per unit volume of reaction mixture taken as the dependent variable. The time dependence of binding was interpreted according to scheme 1, in which the radioligand (P) binds reversibly to distinct and noninterconverting sites (R_j, $j = 1, 2, \ldots n$) with second- and first-order rate constants of k_{+P_j} and k_{-P_j}, respectively.

$$P + R_j \underset{k_{-P_j}}{\overset{k_{+P_j}}{\rightleftharpoons}} PR_j \qquad \text{scheme 1}$$

$$B_{obsd} = ([P]_b + NS[P])SA \qquad (1)$$

where

$$[P]_b = \sum_{j=1}^{n} [PR_j]$$

$$[PR_j] = [PR_j]_{eq}(1 - e^{-(k_{+P_j}[P] + k_{-P_j})t}) \qquad \text{(association)} \quad (1a)$$

or

$$[PR_j] = [PR_j]_{eq}e^{-k_{P_j}t} \qquad \text{(dissociation)} \quad (1b)$$

and

$$[PR_j]_{eq} = \frac{[P][R_j]_t}{[P] + K_{P_j}}$$

$$[R_j]_t = [R_j] + [PR_j]$$

$$[P] \equiv [P]_t$$

Total binding of the radioligand (B_{obsd}, dpm/ml) at any time (t) was calculated according to eq. 1, where $[P]_b$ represents total specific binding of the probe to all sites, NS is the fraction of unbound radioligand ([P]) that appears as nonspecific binding, and SA is the specific radioactivity. With data on the association (eq. 1a) or dissociation (eq. 1b) of [^{3}H]histamine taken separately, K_{P_j} was assigned the value estimated from the concentration dependence of binding at equilibrium (see Table 2B); in the case of association, k_{-P_j} was taken as $K_{P_j}k_{+P_j}$. Association and dissociation were assessed simultaneously in some analyses, and the program selected between eq. 1a and eq. 1b according to a flag encoded with each set of data; single values of k_{+P_j} and k_{-P_j} were common to all of the data, and K_{P_j} was taken as k_{-P_j}/k_{+P_j}. The fitting procedure was coded to optimize the values of $k_{\pm P_j}$, F_j, and $[R]_t$; F_j represents that fraction of all sites corresponding to those of type j (i.e., $F_j = [R_j]_t/[R]_t$, where $[R]_t = \Sigma_{j=1}^{n} [R_j]_t$). The fraction of specific binding associated with each class of sites is shown below as F'_j (i.e., $F'_j = [PR_j]_{eq}/\Sigma_{j=1}^{n} [PR_j]_{eq}$) and was calculated from the parametric values obtained by fitting eq. 1.

Nonspecific binding was independent of time under the conditions of all experiments. The free concentration of [^{3}H]histamine ([P]) was taken as equal to the total concentration ($[P]_t$), because specific binding represented a negligible fraction of the total radioligand in these experiments (i.e., $[P]_b < 0.03[P]_t$). To measure the time dependence of association, [^{3}H]histamine was added to the homogenate at $t = 0$, and the reaction was followed to equilibrium. For dissociation, the radioligand first was equilibrated with the receptor for 4 hr; the time course study then was initiated at $t = 0$ by the addition of 1.0 mM unlabeled histamine.

Data on the concentration dependence of binding at equilibrium were analyzed empirically according to eq. 2, in which n_H is the Hill coefficient and IC_{50} is the concentration of the unlabeled ligand (A) that reduces specific binding by 50%; $B_{A=0}$ and $B_{A=\infty}$ are the values of the function when [A] = 0 and as [A] $\rightarrow \infty$.

$$B_{obsd} = (B_{A=0} - B_{A=\infty}) \frac{IC_{50}^{n_H}}{IC_{50}^{n_H} + [A]^{n_H}} + B_{A=\infty} \qquad (2)$$

Empirical analyses also were carried out according to eq. 3, in which the specific signal reflects n components, each with a Hill coefficient of 1; F'_j is that fraction of specific binding inhibited by the unlabeled ligand ([A]) with potency K_j. Other parameters are as described for eq. 2.

$$B_{obsd} = (B_{A=0} - B_{A=\infty}) \sum_{j=1}^{n} \frac{F'_j K_j}{K_j + [A]} \qquad (3)$$

A mechanistic description of data acquired at equilibrium was obtained in terms of scheme 2, in which [^{3}H]histamine (P) and the unlabeled ligand (A) compete for a mixture of distinct and mutually independent sites (R_j, $j = 1, 2, \ldots n$).

$$PR_j \underset{[P[/K_{P_j}}{\rightleftharpoons} R_j \underset{}{\overset{[A]/K_{A_j}}{\rightleftharpoons}} AR_j \qquad \text{scheme 2}$$

Sites of type j bind P and A with equilibrium dissociation constants K_{P_j} and K_{A_j}, respectively, and constitute the fraction F_j of all sites (i.e., $F_j = [R_j]_t/[R]_t$, where $[R_j]_t = [R_j] + [AR_j] + [PR_j]$ and $[R]_t = \Sigma_{j=1}^{n} [R_j]_t$). The parameters K_{P_j} and K_{A_j} were taken as equal whenever P and A represented the radiolabeled and unlabeled forms of histamine. Estimates of total binding were analyzed according to eq. 4, in which NS and SA are as defined for eq. 1 above.

$$B_{obsd} = \{[P]_b + NS([P]_t - [P]_b)\}SA \qquad (4)$$

where

$$[P]_b = [PR_1] + [PR_2] + \ldots + [PR_n].$$

In analyses with labeled and unlabeled histamine as the only ligands, total specific binding of the probe ($[P]_b$) was calculated as the appropriate root of a quartic polynomial in which P and A appear in units of total concentration (i.e., eq. 67 in Ref. 30); the assumption that K_{P_j} equals K_{A_j} is included in the derivation. In all other analyses, values of $[PR_j]$ were calculated for all j [i.e., $[PR_j] = [P][R_j]_t/([P] + K_{P_j})$] and summed to obtain $[P]_b$; the free concentration of [^{3}H]histamine ([P]) was obtained by solving a set of implicit equations for all reactants (i.e., $[P]_t$, $[A]_t$, and $[R_j]_t$; eqs. 76 and 77 in Ref. 30). The restraint for isotopic dilution (i.e., $K_{P_j} = K_{A_j}$) was applied whenever invoked by a flag encoded with the data.

Statistical procedures. All functions were fitted to experimental data by means of an iterative procedure based on the nonlinear, least-squares algorithm of Marquardt (31). The data were weighted according to the nature of the experiment. In studies on the time dependence of binding and on the inhibition of [^{3}H]histamine by unlabeled ligands, standard errors were an essentially constant percentage of the mean, and the data were weighted accordingly. This approximation is not valid at the lowest concentrations of [^{3}H]histamine used in experiments at graded concentrations of the radioligand. Because all samples were counted to a constant time, and not to a constant error, the counting error is relatively high and predominates under such conditions. The measured error on replicate determinations thus tends to be a higher percentage of the mean than at higher concentrations of the radioligand. All of the data, therefore, were weighted according to the measured error on each point in analyses involving the results of assays at concentrations of [^{3}H]histamine below 0.5 nM. With fits that could be deemed acceptable, neither the sum of squares nor the correlation of neighboring residuals was dominated by the data from one experiment or group of experiments; weighted residuals were of comparable magnitude within single sets of data, and multiple sets of data made comparable contributions to the total sum of squares from simultaneous analyses.

Parametric values obtained from independent analyses of data from two or more experiments were averaged to obtain the mean and standard error. Values obtained from single analyses are presented together with the parametric error as estimated from the diagonal element of the covariance matrix; such estimates reflect the range of values within which the parameter is without appreciable effect on the

sum of squares. In analyses of pooled data, separate values of $[R]_t$ and NS were assigned to data from separate experiments. Single values of $k_{\pm P_j}$, K_{P_j}, and K_{A_j}, and F_j generally were common to all of the data from repeated experiments; further constraints on those parameters are described where appropriate in the text.

The fit of individual expressions has been assessed by testing the significance of the t statistic for the correlation coefficient of neighboring residuals. The effects of more or fewer parameters on the weighted sum of squares were compared by testing the significance of the F statistic. Values of p for certain tests are denoted by subscripts as follows: p_1, the correlation of neighboring residuals for the best fit to a single class of sites (t statistic); p_2, the difference in the weighted sum of squares between best fits with one and two classes of sites (F statistic). Other tests are described where appropriate in the text. Further details regarding the statistical procedures have been reported elsewhere (5, 30).

Results

Kinetic studies. Total binding of [^{3}H]histamine in homogenates of guinea pig cerebral cortex attained a maximum within 2 hr of mixing and remained unchanged for up to 4 hr thereafter (Fig. 1, *inset*). Total binding in the presence of 1.0 mM unlabeled histamine was independent of time over the same period. The global sum of squares is substantially less with two classes of sites than with one when the data from two experiments are analyzed separately in terms of scheme 1 (eq. 1, $p_2 < 0.00001$). Two classes of sites also are required to account for the binding of histamine at equilibrium, as described below; the corresponding estimates of K_{P_j} (eq. 4, $n = 2$) can be used to fix the ratio k_{-P_j}/k_{+P_j} in analyses with eq. 1, and the fitted values of k_{+P_j} are listed in Table 1A.

The net dissociation of [^{3}H]histamine initiated after equilibration over 4 hr is illustrated in Fig. 1. A single exponential is not sufficient to describe the data from four experiments, as indicated by the global correlation of neighboring residuals from separate analyses (eq. 1, $n = 1$, $p_1 < 0.00001$); the fit is significantly better with two exponentials (eq. 1, $n = 2$, $p_1 = 0.84$, $p_2 < 0.00001$), and the mean values of k_{-P_j} are listed in Table 1B. Those estimates are in excellent agreement with the values of k_{-P_1} (0.0092 sec^{-1}) and k_{-P_2} (0.00065 sec^{-1}) that can be inferred from the values of k_{+P_j} and K_{P_j} listed in Tables 1A and 2, respectively (i.e., $k_{-P_j} = k_{+P_j}K_{P_j}$).

The consistency implicit in the rate constants listed in Table 1 does not extend to the relative numbers of sites in each class. Those sites ostensibly of low affinity account for 67% of specific binding when the distribution is defined by net association of the radioligand but only 46% as defined by net dissociation (i.e., F'_2 in Table 1). The difference conflicts with the expectation that the distribution be the same regardless of the experimental protocol; the concentration of the radioligand was 1.2–1.4 nM throughout, and net dissociation was initiated only after the reaction had attained equilibrium.

The level of significance associated with the discrepancy in F'_2 can be tested in analyses of the data on association and dissociation taken together. As expected, there is no increase in the global sum of squares when k_{-P_j} in eq. 1a is defined by data on the time dependence of dissociation (i.e., k_{-P_j} in eq. 1b) rather than by the values of K_{P_j} taken from measurements at equilibrium ($p > 0.1$). The sum of squares is significantly greater with a single value of F_2 than with separate values for association on the one hand and dissociation on the other ($p = 0.00010$); moreover, the increase is not reversed if eq. 1 is fitted with three classes of sites rather than two ($p > 0.1$). It is noteworthy that the combined analyses are self-contained, in contrast to the requirement for an independent estimate of k_{-P_j}/k_{+P_j} in fits of eq. 1a to data on association alone. The four rate constants are not correlated with each other and are defined by a broad but unique minimum in the weighted sum of squares. When association and dissociation are assigned separate values of F_2, the fitted values of k are as follows: $k_{+P_1} = 1.9\ \mu M^{-1}$ sec^{-1}, $k_{-P_1} = 0.0081$ sec^{-1}, $k_{+P_2} = 0.0017\ \mu M^{-1}$ sec^{-1}, and $k_{-P_2} = 0.00071$ sec^{-1}. These results are in excellent agreement with those summarized in Table 1.

Scheme 1 was selected as the simplest mechanism that predicts biexponential kinetics as well as multiple classes of sites at equilibrium; moreover, the observed agreement among estimates of $k_{\pm P_j}$ and K_{P_j} is consistent with the underlying assump-

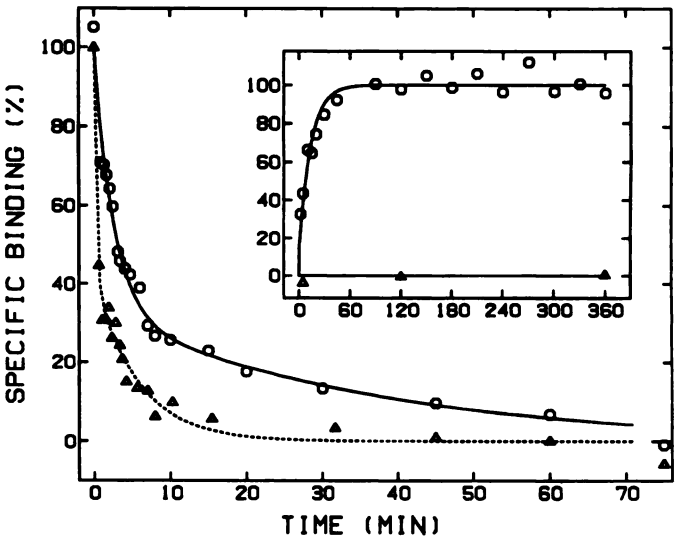

Fig. 1. Time dependence of the binding of [^{3}H]histamine. *Inset*, [^{3}H]histamine was added to homogenized tissue (0.60 mg of protein/ml) either alone (○) or together with unlabeled histamine (△); the final concentrations of the radioligand and the unlabeled analogue were 1.32 nM and 1.0 mM, respectively. Total binding was measured in aliquots removed at the times shown on the *abscissa*. The *lines* represent the best fit of eq. 1 to the data acquired in the absence and presence of unlabeled histamine taken together; the parameters K_{P_1} and K_{P_2} (i.e., k_{-P_j}/k_{+P_j}) were assigned the values listed in Table 2D for binding in the absence of GMP-PNP (log $K_{P_1} = -8.452$, log $K_{P_2} = -6.338$). Parametric values obtained by regression are as follows: $k_{+P_1} = 2.5 \pm 1.3\ \mu M$ sec^{-1}, $k_{+P_2} = 0.0017 \pm 0.0003\ \mu M^{-1}$ sec^{-1}, $F_2 = 0.9944 \pm 0.0020$, and $R_t = 8600 \pm 1060$ pmol/g of protein. R_t was taken as zero for data acquired in the presence of unlabeled histamine. *Main panel*, tritiated histamine was added to homogenized tissue (0.89–1.30 mg of protein/ml) to yield a final concentration of 1.2–1.4 nM. After incubation for 4 hr, aliquots were removed to assay for binding, and unlabeled histamine then was added either alone (○) or together with GMP-PNP (△) to yield final concentrations of 1.0 mM for histamine and 0.1 mM for the nucleotide ($t = 0$). Total binding subsequently was measured in aliquots removed at the times shown on the *abscissa*. The *lines* represent the best fit of eq. 1 to the data. Parametric values obtained by regression are as follows: without GMP-PNP (○), $k_{-P_1} = 0.0079 \pm 0.0023$ sec^{-1}, $k_{-P_2} = 0.00061 \pm 0.00014$ sec^{-1}, $F_2 = 0.9903 \pm 0.0023$ ($F'_2 = 0.33$), and $R_t = 3700 \pm 710$ pmol/g of protein; with GMP-PNP (△), $k_{-P_1} = 0.-58 \pm 0.022$ sec^{-1}, $k_{-P_2} = 0.0029 \pm 0.0006$ sec^{-1}, $F_2 = 0.9847 \pm 0.0046$ ($F'_2 = 0.41$), and $R_t = 2600 \pm 420$ pmol/g of protein. The values of K_{P_1} and K_{P_2} were assigned as described above for the results shown in the *inset*. *Points at the upper limit on the abscissa* (i.e., $t > 70$ min) are from control experiments containing 0.1 mM unlabeled histamine added at the same time as the radioligand; the data were included in the analysis at $t = 600$ min. *Values plotted on the ordinate* have been normalized to the fitted values of eq. 1 at $t = 0$ and as $t \to \infty$ taken as 0 and 100 (*inset*) or as 100 and 0 (*main panel*).

TABLE 1

Kinetically determined parameters for the binding of histamine

The association (A) and the net dissociation (B and C) of [^{3}H]histamine were measured over time at a radioligand concentration of 1.2–1.4 nM, as described in the legend to Fig. 1. Dissociation was initiated by the addition of 1.0 mM unlabeled histamine, either alone (B) or together with 0.1 mM GMP-PNP (C), to samples that had been preequilibrated for 4 hr. The number of experiments is shown in parentheses. Parametric values listed in the table represent best fits of eq. 1 (n = 2); the parameters K_{P_1} and K_{P_2} (i.e., k_{-P_j}/k_{+P_j}) were assigned the values listed in Table 2D (log K_{P_1} = −8.452, log K_{P_2} = −6.338). Rates of association (A) were determined in a simultaneous analysis (eq. 1a) of the data from two experiments; single values of k_{+P_1}, k_{+P_2}, and F_2 were common to both sets of data, and values of [R]$_t$ and NS were assigned separately to each. Rates of dissociation (B and C) were determined in separate analyses (eq. 1b) of the data from three or four experiments; the individual values were averaged to obtain the means ± standard errors listed in the table. The data are illustrated in Fig. 1.

Type of experiment	Protocol	Unlabeled ligands added after 4-hr equilibration	Eq. 1 (n = 2)			F'_2[a]
			k_{+P_1}	k_{+P_2}	F_2	
Association	A		(2) 2.2 ± 0.8 μM^{-1} sec^{-1}	0.0016 ± 0.0002 μM^{-1} sec^{-1}	0.9948 ± 0.0013	0.667 ± 0.001
Dissociation	B	1 mM Histamine	(4) 0.0081 ± 0.0001 sec^{-1}	0.00070 ± 0.0001 sec^{-1}	0.9876 ± 0.0028	0.46 ± 0.06
	C	1 mM Histamine + 0.1 mM GMP-PNP	(3) 0.050 ± 0.012 sec^{-1}	0.0019 ± 0.0004 sec^{-1}	0.9816 ± 0.0022	0.37 ± 0.03

[a] The fraction of specific binding associated with the process corresponding to k_{+P_2} (A) or k_{-P_2} (B and C).

TABLE 2

Parametric values for the binding of histamine at equilibrium

Total binding at graded concentrations of [^{3}H]histamine (A and D) or at 1.2–1.4 nM [^{3}H]histamine and graded concentrations of unlabeled histamine (B–D) was measured in the absence and presence of 0.1 mM GMP-PNP. Further details are described in the legend to Fig. 2. Data from multiple experiments were analyzed simultaneously according to eq. 4 (n = 2); the number of experiments is shown in parentheses. Parametric values were obtained as follows. A, Limits on log K_{P_1}, log K_{P_2}, F_2, $R_{1,t}$, and $R_{2,t}$ were determined by mapping the weighted sum of squares with respect to log K_{P_2}, as described in the text and in the legend to Fig. 3. B, Single values of K_{P_1}, K_{P_2}, and F_2 were common to the four sets of data obtained either in the absence or in the presence of GMP-PNP. Separate values of [R]$_t$ were assigned to each set of data, and the fitted estimates were normalized per g of protein to obtain R_t; $R_{1,t}$ and $R_{2,t}$ were calculated from R_t and F_2, and the individual values were averaged to obtain the means ± standard errors listed in the table. C, Single values of K_{P_1} and K_{P_2} were common to all of the data; single values of [R]$_t$ were assigned as described for the analyses in B. D, Single values of K_{P_1}, K_{P_2}, and F_2 were common to all of the data acquired according to both experimental procedures and under the same conditions with respect to GMP-PNP. Values of [R]$_t$ were assigned as described for the analyses in A and B.

Analysis	Ligand varied	GMP-PNP	Eq. 4 (n = 2)					F'_2[a]
			log K_{P_2}	log K_{P_2}	F_2	$R_{1,t}$	$R_{2,t}$	
		mM				pmol/g	pmol/g	
A	[^{3}H]Histamine (4)	0	−8.68 to −8.44	>−7.85	>0.65	40–90	>120	
	[^{3}H]Histamine (4)	0.1	−8.81 to −8.47	>−7.75	>0.80	20–50	>100	
B	Histamine (4)	0	−8.39 ± 0.02	−6.21 ± 0.14	0.929 ± 0.017	118 ± 9	1550 ± 120	0.103 ± 0.001
	Histamine (4)	0.1	−8.27 ± 0.03	−5.96 ± 0.20	0.958 ± 0.017	78 ± 4	1760 ± 90	0.129 ± 0.001
C	Histamine (4)	0	−8.36 ± 0.02	−6.16 ± 0.11	0.927 ± 0.015	125 ± 10	1600 ± 130	0.095 ± 0.001
	Histamine (4)	0.1			0.954 ± 0.010	65 ± 4	1360 ± 70	0.146 ± 0.001
D	[^{3}H]Histamine (4)	0	−8.45 ± 0.02	−6.34 ± 0.22	0.908 ± 0.039	98 ± 6	986 ± 59	0.095 ± 0.001
	Histamine (4)	0						
	[^{3}H]Histamine (4)	0.1	−8.43 ± 0.03	−6.33 ± 0.32	0.931 ± 0.045	55 ± 3	736 ± 35	0.127 ± 0.002
	Histamine (4)	0.1						

[a] The fraction of specific binding associated with K_{P_2}. Eq. 3 (n = 2) was fitted to simulated data computed for each experiment according to eq. 4; the errors reflect the variation in the concentration of [^{3}H]histamine from experiment to experiment.

tion that association and dissociation are one-step processes. Taken together with the behavior of the system at equilibrium (see below), the discrepancy in F_2 argues against the notion of independent and noninterconverting sites. Taken alone, however, the time dependence of binding is mechanistically ambiguous. Even a single class of sites can exhibit multiexponential kinetics if there is isomerization of the ligand-receptor complex (e.g., P + R ⇌ PR ⇌ PR*), and the number of processes is commensurately greater when two or more classes are involved.

Dissociation of the radioligand exhibits a noncompetitive sensitivity to guanyl nucleotides. Initiation of dissociation with 1.0 mM histamine plus 0.1 mM GMP-PNP rather than with histamine alone increased k_{-P_1} and k_{-P_2} by about six-fold and three-fold, respectively (Fig. 1; Table 1C). The changes are significant, as indicated by the increase in the sum of squares when data acquired with and without the nucleotide are analyzed with single rather than separate values of k_{-P_1} ($p < 0.00001$), k_{-P_2} ($p = 0.017$), or both ($p < 0.00001$). An increase in the rate of dissociation at both classes of sites suggests that both states are sensitive to GMP-PNP, which thus appears to favor an unobserved state of still lower affinity. The nucleotide alone reduces but does not eliminate measurable binding at equilibrium; it therefore is possible to incubate the membranes with both GMP-PNP and the radioligand and then to study the dissociation kinetics of the residual binding. Under those conditions, estimates of k_{-P_1} and k_{-P_2} are greater than those obtained in the absence of GMP-PNP; the ratio of signal to noise is small, however, and the differences are not significant.

Binding at equilibrium. Total binding at graded concentrations of [^{3}H]histamine is illustrated in Fig. 2A, and the parametric values from analyses in terms of scheme 2 (eq. 4) are summarized in Table 2. A single class of sites is inadequate to describe binding either with or without GMP-PNP, as indicated by the global correlation of neighboring residuals from independent analyses of the data from four experiments ($p_1 < 0.00001$). The addition of a second class yields a significant reduction in the sum of squares ($p_2 < 0.00001$), but K_{P_2} and [R]$_t$ are highly correlated owing to the upper limit on practicable concentrations of [^{3}H]histamine; neither parameter is defined explicitly by the data.

To establish limits on the various parameters (Table 2A), the weighted sum of squares was mapped with respect to K_{P_2}. The value of log K_{P_2} is defined by a shallow minimum at −7.38

in the absence of GMP-PNP (Fig. 3A), but any value greater than −7.85 (i.e., $K_{P_2} > 14$ nM) yields satisfactory agreement between the model and the data ($p \geq 0.05$). The value of $[R_2]_t$ is defined only by an asymptotic minimum at low values of K_{P_2} (Fig. 3B); $[R_1]_t$ lies between a minimum at the lowest acceptable value of K_P and an asymptotic maximum when

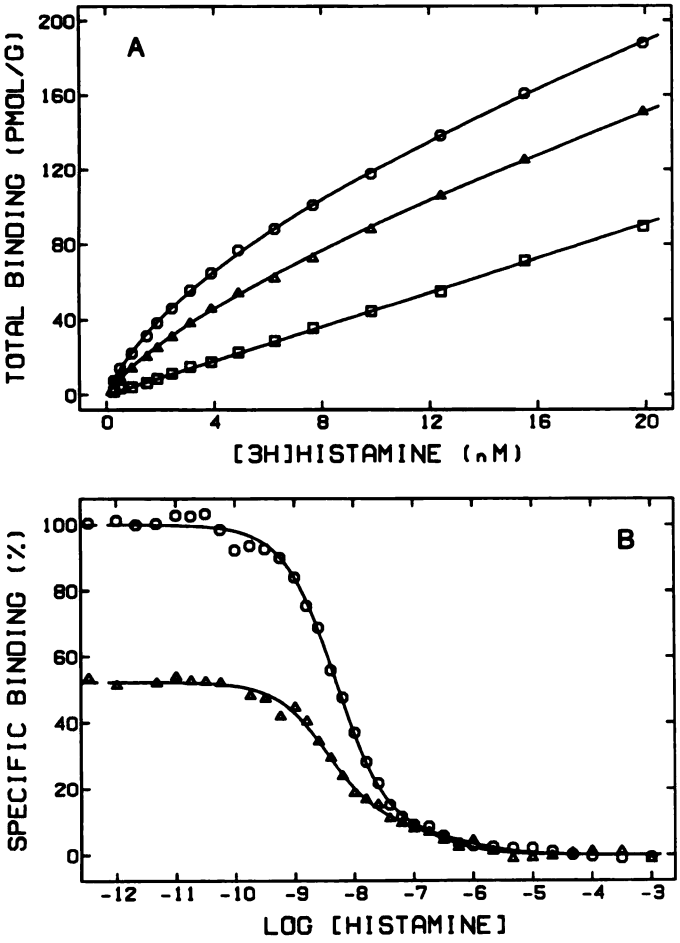

Fig. 2. Binding of histamine with and without GMP-PNP. A, Total binding at graded concentrations of [^{3}H]histamine was measured after incubation of the membranes (1.16 mg of protein/ml) with [^{3}H]histamine alone (○), in the presence of 0.1 mM GMP-PNP (△), or in the presence of 1.0 mM unlabeled histamine (□). The three sets of data were acquired in parallel using aliquots of the same homogenate. The *lines* represent the best fit of eq. 4 ($n = 2$); separate values of K_{P_1}, K_{P_2}, F_2, and $[R]_t$ were assigned to data acquired in the absence and presence of the nucleotide, and a single value of *NS* was common to all of the data. Parametric values obtained by regression are as follows: without GMP-PNP, $K_{P_1} = 2.1 \pm 0.9$ nM, $K_{P_2} > 2$- nM, $R_{1,t} = 35$–50 pmol/g of protein, and $R_{2,t} > 100$ pmol/g of protein; with GMP-PNP, $K_{P_1} = 2.6 \pm 0.5$ nM, $K_{P_2} > 11$ nM, $R_{1,t} = 5$–10 pmol/g of protein, and $R_{2,t} > 100$ pmol/g of protein. B, Total binding was measured after incubation of the membranes (0.68 mg of protein/ml) with [^{3}H]histamine (1.29 nM) and graded concentrations of unlabeled histamine in the absence of guanyl nucleotide (○) or in the presence of 0.1 mM GMP-PNP (△). The two sets of data were acquired in parallel using aliquots of the same homogenate. The *lines* represent the best fits of eq. 4 ($n = 2$), and the parametric values obtained by regression are as follows: without GMP-PNP, log $K_{P_1} = -8.46 \pm 0.03$, log $K_{P_2} = -5.98 \pm 0.29$, $F_2 = 0.932 \pm 0.039$ ($F'_2 = 0.055 \pm 0.010$ in eq. 3), and $R_t = 1780 \pm 1060$ pmol/g of protein; with GMP-PNP, log $K_{P_1} = -8.66 \pm 0.07$, log $K_{P_2} = -6.54 \pm 0.19$, $F_2 = 0.947 \pm 0$l016 ($F'_2 = 0.177 \pm 0.028$ in eq. 3), and $R_t = 755 \pm 270$ pmol/g of protein. *Values plotted on the ordinate* have been normalized at 100 and 0 to the asymptotic values of eq. 4 for binding in the absence of GMP-PNP. *Points at the lower end of the abscissa* indicate binding in the absence of unlabeled histamine.

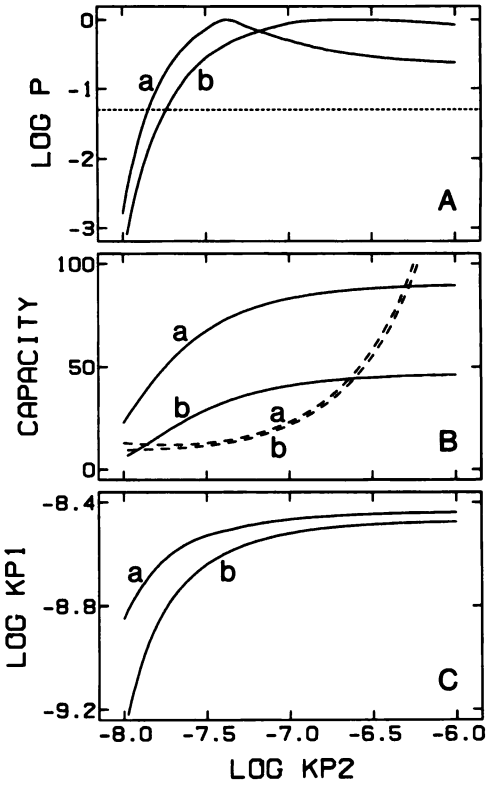

Fig. 3. Limits on parametric values for the binding of [^{3}H]histamine. Eq. 4 ($n = 2$) was fitted to the data from four experiments of the type illustrated in Fig. 2A. Two series of analyses were performed in which the estimates of total binding obtained with the radioligand either alone (*a*) or in the presence of GMP-PNP (*b*) were paired with the corresponding estimates of nonspecific binding, obtained in the presence of 0.1 mM unlabeled histamine. Separate values of K_{P_1}, F_2, $[R]_t$, and *NS* were assigned to the data from each experiment, and a single value of log K_{P_2} was fixed successively at different values over the range shown on the *abscissa*. A, The global sum of squares from each fit was compared with that from the best fit to obtain the *F* statistic and the corresponding value of log *P* plotted on the *ordinate*. . . ., 95% level of confidence. B, Individual values of $R_{1,t}$ and $R_{2,t}$ (pmol/g of protein) were calculated from the fitted estimates of F_2 and $[R]_t$, and the results from the four experiments were averaged to obtain the means plotted on the *ordinate* (——, $R_{1,t}$; – – –, $R_{2,t}$). The scale for $R_{2,t}$ is 10 times that shown (i.e., 0–1000 pmol/g of protein). C, The fitted values of log K_{P_1} from the four experiments were averaged to obtain the means plotted on the *ordinate*.

K_{P_2} is large (Fig. 3B). Estimates of log K_{P_1} are essentially independent of the value of K_{P_2} when the latter exceeds about 14 nM, and the range of possible values is from −8.68 to −8.44 (Fig. 3C; Table 2A). GMP-PNP reduced the specific binding of [^{3}H]histamine by approximately 50% at all concentrations of the radioligand (Fig. 2A). The overall pattern is similar to that found in the absence of nucleotide, which effected a 40–50% reduction in $[R_1]_t$ with little if any change in K_{P_1}.

Generally consistent results were obtained by isotopic dilution at a radioligand concentration of 1.2–1.4 nM (Fig. 2B). The mean Hill coefficients from four experiments are 0.83 ± 0.02 and 0.76 ± 0.05 for data acquired in the absence and presence of GMP-PNP, respectively. In either case, two classes of sites are required for agreement with eq. 4 ($p_1 < 0.00001$, $p_2 < 0.00001$). In contrast to the situation with graded concentrations of the radioligand, both K_{P_1} and K_{P_2} are defined by a narrow minimum in the sum of squares. The parameter $[R]_t$ remains correlated with K_{P_2}, but the former is known to within a range of uncertainty defined by the latter. The four sets of

data acquired either with or without GMP-PNP can be combined, and the parametric values obtained with single values of K_{P_1}, K_{P_2}, and F_2 are listed in Table 2B. The nucleotide decreased $R_{1,t}$ with little effect on K_{P_1}. There was a 1.14-fold increase in $R_{2,t}$, but the change is difficult to interpret in light of a concomitant 1.78-fold increase in K_{P_2} and the correlations among K_{P_2}, F_2, and $[R]_t$. There is little effect on the sum of squares if nucleotide-related changes in K_{P_j} are assumed to represent experimental error and single values are assigned to all of the data ($p = 0.079$). The fitted estimates of affinity and capacity are listed in Table 2C. When estimated in this manner, both $R_{1,t}$ and $R_{2,t}$ are reduced in the presence of GMP-PNP.

Data acquired at graded concentrations of labeled and unlabeled histamine can be pooled to yield the parametric values listed in Table 2D. No assumption was made regarding the effects of GMP-PNP, which appears to decrease both $R_{1,t}$ and $R_{2,t}$ with no appreciable change in the corresponding values of K_{P_j}. The combined analysis is essentially without effect on the sum of squares obtained for data acquired in the absence of GMP-PNP ($p = 0.066$); there was a small increase in the presence of GMP-PNP ($p = 0.0073$), reflecting the 1.6-fold discrepancy in K_{P_1} between the value obtained by isotopic dilution and the upper limit defined by graded concentrations of the radioligand (see Table 2, A and B).

Pharmacological characterization. Twenty-five histaminic ligands were tested for their inhibitory effect on the specific binding of 1.2–1.4 nM [^{3}H]histamine, and estimates of the Hill coefficient vary from 0.66 with burimamide to 1.54 with pyridylethylamine (Table 3). Thirteen compounds require only a single class of sites for agreement between the data and best fits of eq. 3; either the global correlation of neighboring residuals is not significant ($p_1 > 0.05$) or the global sum of squares is not appreciably less with two classes of sites rather than one ($p_2 > 0.05$). Twelve compounds reveal Hill coefficients less than about 0.94 and require at least two classes of sites ($p_1 \leq 0.00051$; $p_2 \leq 0.00004$). There is no further reduction for any compound if eq. 3 is fitted with three classes of sites rather than two. Representative data are illustrated in Fig. 4. All compounds inhibited the specific binding of [^{3}H]histamine to the same level. Estimates of total binding in the presence of 1.0 mM unlabeled histamine differ from the fitted asymptote of eq. 3 ($B_{A=\infty}$) by an amount that typically represents <2% of inhibitable binding ($B_{A=0} - B_{A=\infty}$). All of the compounds tested therefore appear to block access of the radioligand to the same sites.

Among those compounds that reveal two classes of sites, mean values of F'_2 vary from 0.098 with the agonist N^{α}-methylhistamine to 0.85 with the antagonist cimetidine. Values of log (K_2/K_1) vary from 0.73 with SK&F 92540 to 2.62 with N^{α}-methylhistamine and 2.77 with cimetidine (Table 3). There is a general tendency for the apparent distribution of sites to be weighted in favor of one state or the other when the apparent affinities for the two states differ by >10-fold; the sites are equally distributed when the difference in affinity is 10-fold or less (Fig. 5). Also, H_2 and H_3 agonists favor the state of high affinity, whereas antagonists tend to favor the state of low affinity; the H_2 agonist and H_3 antagonist imidazolylpropylguanidine is ambivalent.

Ligand-dependent differences in F'_2 are inconsistent with the notion of noninterconverting sites. The values of K_1, K_2, and F'_2 tend to be correlated, however, and the significance of the difference therefore was tested for all pairs of compounds. Data from replicated experiments with two ligands were fitted with a single value of F'_2 (eq. 3, $n = 2$), and the sum of squares was compared with that obtained with two values, one for each ligand; values of K_1 and K_2 were assigned separately to the data from each experiment. The levels of significance for the difference in the sum of squares are shown in Table 4. Among the 55 possible combinations, the difference in F'_2 is highly significant in 27 ($p < 0.001$) and somewhat less so in 11 ($0.001 < p < 0.05$). The largest differences tend to be those between agonists and antagonists, as illustrated in Fig. 5 and by the values of p listed in Table 4; differences among agonists alone or among antagonists alone are somewhat smaller, but they nevertheless are significant in three of six combinations of the former and in 10 of 15 combinations of the latter ($p < 0.05$).

The comparisons summarized in Table 4 are based on the assumption that all ligands show the same preference for the two states. In the analyses with single values of F'_2, initial estimates of K_1 and K_2 approximated the values listed in Table 3; in every case, convergence was to a local minimum such that K_2 exceeded K_1 for both ligands being compared. A different arrangement is suggested by the pattern illustrated in Fig. 5, where it appears that agonists and antagonists favor different states. The results summarized in Table 5 are from an alternative series of analyses in which the starting values of K_1 and K_2 implied an opposite preference of agonists ($K_1 < K_2$) and antagonists ($K_2 < K_1$) for the two classes of sites. Convergence was to a lower minimum in the sum of squares for most pairs of compounds, and the number of significant differences in F'_2 ($P < 0.05$) is reduced from 18 to 14 of 24; with five pairs of compounds the difference is substantially less but remains significant ($0.05 < p < 0.001$).

The foregoing comparisons suggest that agonists and antagonists favor different states of the labeled sites; moreover, the state of low affinity for agonists appears to be of high affinity for antagonists and vice versa. Values of F'_2 generally are indistinguishable with pairs of compounds for which the absolute values of log (K_2/K_1) are similar or the same; significant differences are found with compounds that differ in their ability to discriminate between the two states. The binding parameters thus reveal a symmetry in which agonists show a correlation between log (K_2/K_1) and the apparent fraction of high affinity sites (H_2, $r = 0.94$, $p = 0.0053$, $n = 6$; H_3, $r = 0.93$, $p = 0.022$, $n = 5$) and antagonists show a correlation between log (K_1/K_2) and the apparent fraction of low affinity sites (H_2, $r = 0.84$, $p = 0.038$, $n = 4$; H_3, $r = 0.85$, $p = 0.016$, $n = 5$) (Fig. 5). These relationships suggest that two hyperbolic terms are an appropriate, if empirical, basis on which to describe the data.

Discussion

Nature and identity of the labeled sites. Washed membranes from guinea pig cerebral cortex contain at least two classes of sites differentiated by histamine and other histaminic ligands. The time course of binding is biexponential, both for the attainment of equilibrium and for the net dissociation of [^{3}H]histamine from pre-equilibrated samples. Similarly, the concentration dependence of binding at equilibrium reveals Hill coefficients appreciably less than 1; two classes of sites are required to describe the binding of [^{3}H]histamine itself as well as the inhibitory effect of unlabeled histamine and 11 other histaminic ligands. The effects of GMP-PNP suggest that most

TABLE 3

Parametric values for the inhibition of [^{3}H]histamine by histaminic ligands

Eq. 2 and eq. 3 (n = 1 or 2) were fitted to the data from individual experiments on the binding of [^{3}H]histamine (1.21–1.38 nM) at graded concentrations of the unlabeled ligand; the number of experiments is shown in parentheses following the name of the ligand. The fitted estimates of log K_1, log K_2, and F_2 from each experiment were used to calculate the corresponding values of log IC_{50} and log (K_2/K_1). Individual values of each parameter were averaged to obtain the means ± standard errors listed in the table. Functional potencies for the effects at H_2 and H_3 receptors are listed together with the corresponding references, shown in parentheses; H_2 potencies are for the chronotropic response of guinea pig right atrium, and H_3 potencies are for the release of histamine from presynaptic stores in rat cortex. The data and fitted curves for six compounds are illustrated in Fig. 4.

No.	Unlabeled ligand	H_2 potency (−log EC_{50} or pA_2)	H_3 potency (−log EC_{50} or pA_2)	Eq. 2, n_H	Eq. 3				
					−log K_1	−log K_2	F_2	−log IC_{50}	log (K_2/K_1)
	H_2 and H_3 agonists								
1	Pyridylethylamine (2)	4.36 (32)	*b*	1.54 ± 0.08	4.23 ± 0.04		*a*	4.23 ± 0.04	
2	(*R*)-α-Methylhistamine (2)	3.96 (21)	8.40 (21)	1.13 ± 0.14	9.07 ± 0.25		*a*	9.07 ± 0.25	
3	Thiazolylethylamine (2)	4.30 (32)	<3.10 (19)	1.09 ± 0.02	3.82 ± 0.01		*a*	3.82 ± 0.01	
4	(*R*)-(−)-N^{α}-Methyl-α-chloromethylhistamine (3)	5.67 (33)	2.96 (33)	0.99 ± 0.03	6.70 ± 0.12		*a*	6.70 ± 0.12	
5	Nordimaprit (2)	3.96[c]	*b*	0.99 ± 0.08	5.74 ± 0.07		*a*	5.74 ± 0.07	
6	2-Methylhistamine (2)	4.60 (34)	<3.10 (19)	0.96 ± 0.01	4.16 ± 0.02		*a*	4.16 ± 0.02	
7	(*S*)-(+)-N^{α}-Methyl-α-chloromethylhistamine (2)	4.81 (33)	5.26 (33)	0.95 ± 0.10	7.26 ± 0.02		*a*	7.26 ± 0.02	
8	(*R*)-(+)-α,N^{α}-Dimethylhistamine (2)	3.56 (33)	5.82 (33)	0.91 ± 0.02	7.41 ± 0.03		*a*	7.41 ± 0.03	
9	4-Methylhistamine (5)	5.51 (35)	<3.10 (19)	0.94 ± 0.05	5.31 ± 0.06		*a*	5.31 ± 0.06	
10	(*S*)-(−)-α,N^{α}-Dimethylhistamine (2)	5.96 (33)	4.31 (33)	0.90 ± 0.01	7.12 ± 0.04	6.39 ± 0.04	0.54 ± 0.18	6.69 ± 0.19	0.73 ± 0.06
11	N^{α}-Methylhistamine (5)	5.82 (34)	7.82 (18)	0.85 ± 0.05	8.93 ± 0.04	6.31 ± 0.66	0.098 ± 0.02	8.83 ± 0.04	2.62 ± 0.66
12	Histamine (4)	5.96 (35)	7.21 (18)	0.81 ± 0.03	8.25 ± 0.06	6.18 ± 0.16	0.097 ± 0.02	8.16 ± 0.05	2.07 ± 0.16
13	N^{α},N^{α}-Dimethylhistamine (4)	5.67 (34)	7.62 (19)	0.80 ± 0.01	8.42 ± 0.12	6.55 ± 0.63	0.30 ± 0.13	8.13 ± 0.04	1.87 ± 0.64
14	Dimaprit (6)	5.81 (36)	<3.10 (19)	0.85 ± 0.05	6.50 ± 0.07	5.00 ± 0.40	0.27 ± 0.07	6.27 ± 0.07	1.50 ± 0.41
	H_2 and H_3 antagonists								
15	SK&F 93479 (2)	7.78[c]	*b*	1.26 ± 0.03	4.44 ± 0.04		*a*	4.44 ± 0.04	
16	Tiotidine (2)	7.43 (8)	4.77 (19)	0.94 ± 0.09	3.54 ± 0.04		*a*	3.54 ± 0.04	
17	SK&F 92374 (3)	3.75[c]	*b*	0.93 ± 0.03	5.58 ± 0.01		*a*	5.58 ± 0.01	
18	SK&F 92540 (4)	6.70[c]	*b*	0.87 ± 0.02	5.24 ± 0.11	4.50 ± 0.16	0.48 ± 0.18	4.86 ± 0.03	0.74 ± 0.19
19	(*S*)-(+)-Sopromidine (6)	5.63 (28)	7.35 (33)	0.78 ± 0.03	6.99 ± 0.19	5.84 ± 0.14	0.51 ± 0.10	6.36 ± 0.02	1.15 ± 0.23
20	Cimetidine (3)	6.55 (37)	4.48 (19)	0.73 ± 0.08	6.36 ± 0.56	3.59 ± 0.15	0.85 ± 0.04	3.75 ± 0.12	2.77 ± 0.58
21	Ranitidine (2)	7.20 (37)	<5.92 (19)	0.70 ± 0.06	5.39 ± 0.40	3.53 ± 0.20	0.77 ± 0.07	3.76 ± 0.27	1.86 ± 0.44
22	Norburimamide (2)	3.90 (35)	6.10 (20)	0.68 ± 0.03	7.64 ± 0.15	5.97 ± 0.04	0.71 ± 0.00	6.31 ± 0.05	1.67 ± 0.16
23	Burimamide (2)	5.11 (35)	7.15 (19)	0.66 ± 0.00	8.18 ± 0.27	6.65 ± 0.28	0.44 ± 0.15	7.54 ± 0.05	1.53 ± 0.39
	H_2 agonists and H_3 antagonists								
24	Impromidine (2)	7.64 (38)	7.19 (19)	1.06 ± 0.04	6.92 ± 0.11		*a*	6.92 ± 0.11	
25	(*R*)-(−)-Sopromidine (3)	6.82 (28)	7.25 (33)	0.96 ± 0.01	7.06 ± 0.12		*a*	7.06 ± 0.12	
26	Imidazolylpropylguanidine (2)	5.62 (39)	7.05 (20)	0.78 ± 0.07	8.89 ± 0.22	7.89 ± 0.01	0.52 ± 0.02	8.35 ± 0.08	1.00 ± 0.22

[a] F_2 was fixed at zero.
[b] Not reported.
[c] C. R. Ganellin, University College, London, personal communication.

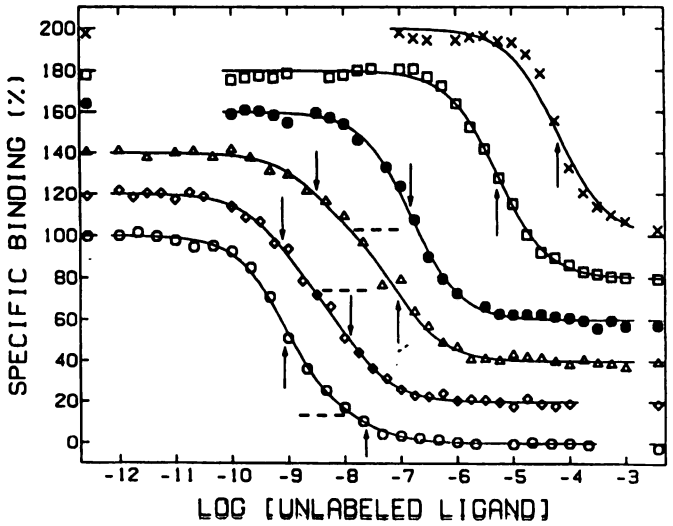

Fig. 4. Inhibition of [^{3}H]histamine by histaminic agonists and antagonists. Total binding was measured after incubation of the membranes with [^{3}H]histamine (1.29–1.36 nM) and with the unlabeled ligand at the concentrations shown on the *abscissa*. The individual compounds are as follows: N^{α}-methylhistamine (○), imidazolylpropylguanidine (◇), burimamide (△), impromidine (●), 4-methylhistamine (□), and pyridylethylamine (X). The *lines* represent best fits of eq. 3, with the number of classes of sites taken as two (N^{α}-methylhistamine, imidazolylpropylguanidine, and burimamide) or one (impromidine, 4-methylhistamine, and pyridylethylamine). *Values plotted on the ordinate* have been normalized to the asymptotic values of eq. 3 taken as 100 and 0; successive *curves* have been offset by 20% for clarity. *Points plotted at the lower end of the abscissa* represent total binding in the absence of unlabeled ligand; *points plotted at the upper end* represent total binding in the presence of 1.0 mM unlabeled histamine and were omitted from the analysis. *Horizontal dashed lines*, the value of F'_2; *arrows*, log K_i.

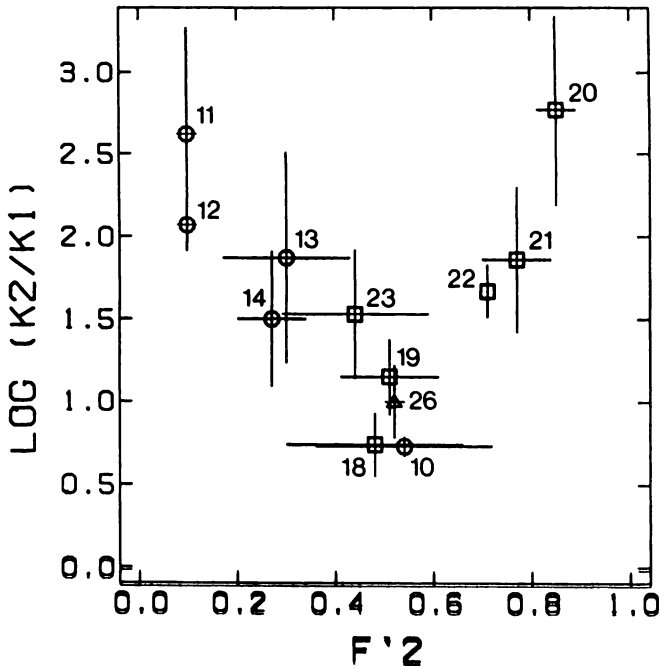

Fig. 5. Comparison of relative potency and apparent distribution of sites. The values of log (K_2/K_1) and F'_2 are listed in Table 3, and the *points* are numbered accordingly. Agonists (○) show a positive correlation between log (K_2/K_1) and that fraction of labeled sites apparently of high affinity for the unlabeled ligand (i.e., F'_1) (H_2, $r = 0.940$, $p = 0.0053$, $n = 6$; H_3, $r = 0.930$, $p = 0.022$, $n = 5$); antagonists (□) show a positive correlation with that fraction apparently of low affinity (i.e., F'_2) (H_2, $r = 0.940$, $p = 0.0053$, $n = 6$; H_3, $r = 0.930$, $p = 0.022$, $n = 5$); antagonists (□) show a positive correlation with that fraction apparently of low affinity (i.e., F'_2) (H_2, $r = 0.837$, $p = 0.038$, $n = 4$; H_3, $r = 0.846$, $p = 0.016$, $n = 5$). Imidazolylpropylguanidine (△) is an H_2 agonist and an H_3 antagonist.

and probably all of the labeled sites are linked to G proteins. Net dissociation of the radioligand remains biphasic when the nucleotide is included with unlabeled histamine, but the rate constants of both processes are increased. Also, inclusion of the nucleotide effects an apparent loss of sites from both classes identified in the binding of histamine at equilibrium.

Guinea pig cortex has been shown to contain H_1 receptors labeled by [^{3}H]mepyramine (40), H_2 receptors labeled by [^{3}H]tiotidine (8) or [^{125}I]iodoaminopotentidine (11), and putative H_3 receptors labeled by N^{α}-[^{3}H]methylhistamine (16). That heterogeneity, together with the failure of GMP-PNP to effect a complete interconversion of sites from higher to lower affinity, raises the possibility that [^{3}H]histamine labels a mixture of gene products in native membranes. It seems unlikely that H_1 receptors constitute an appreciable fraction of the labeled sites in the present investigation. The capacity of cortical membranes for [^{3}H]mepyramine is approximately 100 pmol/g of protein in guinea pig, and the apparent affinity for histamine exceeds 1 μM (40); accordingly, the number of mepyramine-specific sites labeled by [^{3}H]histamine is expected to be unobservably small. Moreover,the apparent affinity of mepyramine for the [^{3}H]histamine-labeled sites was found to be 20 μM in the present investigation, or at least 25,000 times greater than that for H_1 receptors.

The potential contribution of H_2 and H_3 receptors is less steadily assessed. As described below, the inhibitory behavior of 26 histaminic ligands has revealed an enigmatic pattern that is neither H_2 nor H_3 but nonetheless is reminiscent of both. It might be expected, however, that a mixture of those sites and perhaps others yet unidentified ought to behave in a manner consistent with schemes 1 and 2. In particular, the relative number of sites in one state or the other ought to be constant. Contrary to expectation, the apparent distribution of sites between states depends both on the nature of the experiment and on the ligands present. Data on the time dependence of binding yield estimates of F_2 that differ significantly between association on the one hand and dissociation on the other; moreover, the kinetically defined estimates of F_2 (eq. 1) exceed that defined by isotopic dilution at equilibrium (eq. 4). There is wide variability among the values of F'_2 estimated from the inhibitory behavior of 11 unlabeled ligands (eq. 3), and the data reveal a pharmacologically relevant trend (Fig. 5).

Values of F'_2 defined by unlabeled ligands at equilibrium are not definitive, in that all of the differences assessed in Table 4 can be resolved by assuming a degree of heterogeneity greater than two. This question is addressed in the following report (22), where eq. 4 has been used to describe the inhibitory behavior of 13 ligands at two concentrations of [^{3}H]histamine. The radioligand serves as an internal standard in such experiments, in contrast to the situation when all binding is measured at a single concentration of [^{3}H]histamine. Owing to that additional constraint, ligand-dependent differences in K_P and F_i persist with values of n at which the analysis is severely underdetermined. It follows that the multiple states of affinity recognized by histamine and other ligands cannot arise wholly from a mixture of distinct and mutually independent sites (i.e., scheme 2), irrespective of the number of classes assumed to be present.

TABLE 4

Comparison of F'_2 among compounds revealing two classes of sites

Values listed in the table indicate the levels of significance (p) for differences in F'_2 (eq. 3) between pairs of ligands that revealed two classes of sites (Table 3). The number of experiments is shown in parentheses. Further details are described in the text. In those analyses with a single value of F'_2 for both ligands, the starting values of log K_1 and log K_2 were selected on the assumption that agonists and antagonists exhibit the same preference for the two forms of the receptor (i.e., $K_1 < K_2$)

No.	p value									
	H_2 and H_3 antagonists							H_2 and H_3 agonists		
	Imidazolylpropylguanidine	Burimamide	Norburimamide	Ranitidine	Cimetidine	(S)-(+)-Sopromidine	SK&F 92540	Dimaprit	N^{α},N^{α}-Dimethylhistamine	N^{α}-Methylhistamine
10 (S)-(−)-α,N^{α}-Dimethylhistamine (2)	0.13	0.76	0.16	0.16	0.12	0.21	0.087	0.060	0.039	0.056
11 N^{α}-Methylhistamine (5)	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.00005	0.27	<0.00001	
13 N^{α},N^{α}-Dimethylhistamine (4)	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.00060	0.0074		
14 Dimaprit (6)	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.00011			
18 SK&F 92540 (4)	0.018	0.015	0.74	0.23	0.14	0.046				
19 (S)-(+)-Sopromidine (6)	0.78	0.43	0.017	0.0015	0.00012					
20 Cimetidine (3)	0.00029	<0.00001	0.00053	0.41						
21 Ranitidine (2)	0.0015	<0.00001	0.0048							
22 Norburimamide (2)	0.017	<0.00001								
23 Burimamide (2)	0.43									

TABLE 5

Comparison of F'_2 between agonists and antagonists revealing two classes of sites

Values listed in the table indicate the level of significance (p) for differences in F'_2 (eq. 3) between mixed pairs of agonists and antagonists (see Table 4). In those analyses with a single value of F'_2 for both ligands, the starting values of log K_1 and log K_2 implied that the preference for the two forms of the receptor is opposite for agonists ($K_1 < K_2$) and for antagonists ($K_2 < K_1$).

No.		p value					
		Burimamide	Norburimamide	Ranitidine	Cimetidine	(S)-(+)-Sopromidine	SK&F 92540
10	(S)-(−)-α,N^{α}-Dimethylhistamine	0.59	0.47	0.24	0.20	0.77	0.34
11	N^{α}-Methylhistamine	<0.00001	<0.00001	0.28	0.19	<0.00001	0.038
13	N^{α},N^{α}-Dimethylhistamine	<0.00001	<0.00001	0.0018	0.0062	<0.00001	0.032
14	Dimaprit	<0.00001	<0.00001	0.36	0.65	<0.00001	0.029

Status as H_2 receptors. Nine H_2 antagonists reveal a pharmacological specificity distinct from that characteristic of H_2 receptors. Estimates of inhibitory potency at the labeled sites and functional potency in the right atrium fail to agree, irrespective of whether the former is taken as IC_{50} for all compounds (Fig. 6B) or either value of K_j in the case of those compounds that require two classes of sites. This lack of agreement argues against the presence of H_2 receptors, but several lines of evidence favor a more qualified interpretation. Among the H_2 agonists tested, reported estimates of the functional potency in guinea pig right atrium approximate or exceed the corresponding values of K_1 listed in Table 3 (Fig. 6A). Sites of low affinity are observable for six of the nine compounds for which EC_{50} exceeds K_1 by 14-fold or more, and K_2 agrees with EC_{50} to within 8-fold or less for five of the six. An H_2 pharmacological specificity thus emerges for 12 agonists when the appropriate state is identified in the binding assay (Fig. 6A; $r = 0.851$, $p = 0.00045$). Among the five compounds excluded from the comparison, EC_{50} exceeds K_1 by at least 60-fold; because values of F'_2 vary substantially from ligand to ligand, the differences may arise from an inability to observe the relevant state of affinity.

Evidence for H_2 specificity also is found in the preference of H_2 agonists and antagonists for what appear to be different states of the labeled sites. Empirical analyses of data obtained at a low concentration of [^{3}H]histamine suggest that the propensity of the receptor to reveal one state or the other depends upon the difference in the affinity of the ligand for each; moreover, agonists seem to promote one state, whereas antagonists promote the other (Fig. 5). A related but mechanistically explicit pattern is presented in the following paper (22), where the data permit the affinity of [^{3}H]histamine and the unlabeled ligand to be estimated concomitantly in terms of scheme 2 (eq. 4). Six of the seven H_2 agonists show the same apparent preference as histamine for two classes of sites (i.e., $K_{A_1} < K_{A_2}$), whereas the five antagonists show the opposite preference (i.e., $K_{A_2} < K_{A_1}$) (Table 2 in Ref. 22). Of particular interest are the compounds (R)-(−)-sopromidine and imidazolylpropylguanidine, which are agonists at H_2 receptors but antagonists at H_3 receptors. Based on the values of K_{P_j} and K_{A_j}, the preference of each compound for the labeled sites is the same as that of other H_2 agonists.

In an earlier study on the binding of [^{3}H]histamine to cortical membranes from rat (5–7), the pharmacological specificity defined by H_2 antagonists was unequivocally H_2 (6). Those data are in contrast to the present results from guinea pig cortex, but a number of properties are common to both preparations. Histamine itself was found to recognize two classes of sites in the rat, at least under some conditions, and GMP-PNP reduced specific binding of the radioligand by 35–50% (5). The binding profiles of most H_2 agonists were manifestly biphasic (i.e., $K_2/K_1 > 10^3$, $0.9 < F'_2 < 0.4$, eq. 3) (7), in contrast to the relatively steep curves obtained in the present investigation ($K_2/K_1 < 10^3$; Table 3), but the comparison in Fig. 6C demonstrates that inhibitory potency in the guinea pig (log IC_{50}) is in excellent agreement with the values of log K_2 reported previously for the rat ($r = 0.959$, $p = 0.00001$); the agreement is somewhat poorer if the data from rat cortex are compared with either log K_1 ($r = 0.946$, $p = 0.00003$) or log K_2 ($r = 0.862$, $p = 0.0013$) for those agonists that reveal two classes of sites in guinea pig.

The sites in guinea pig cortex closely resemble those in a digitonin-solubilized preparation from rat cortex; the latter also

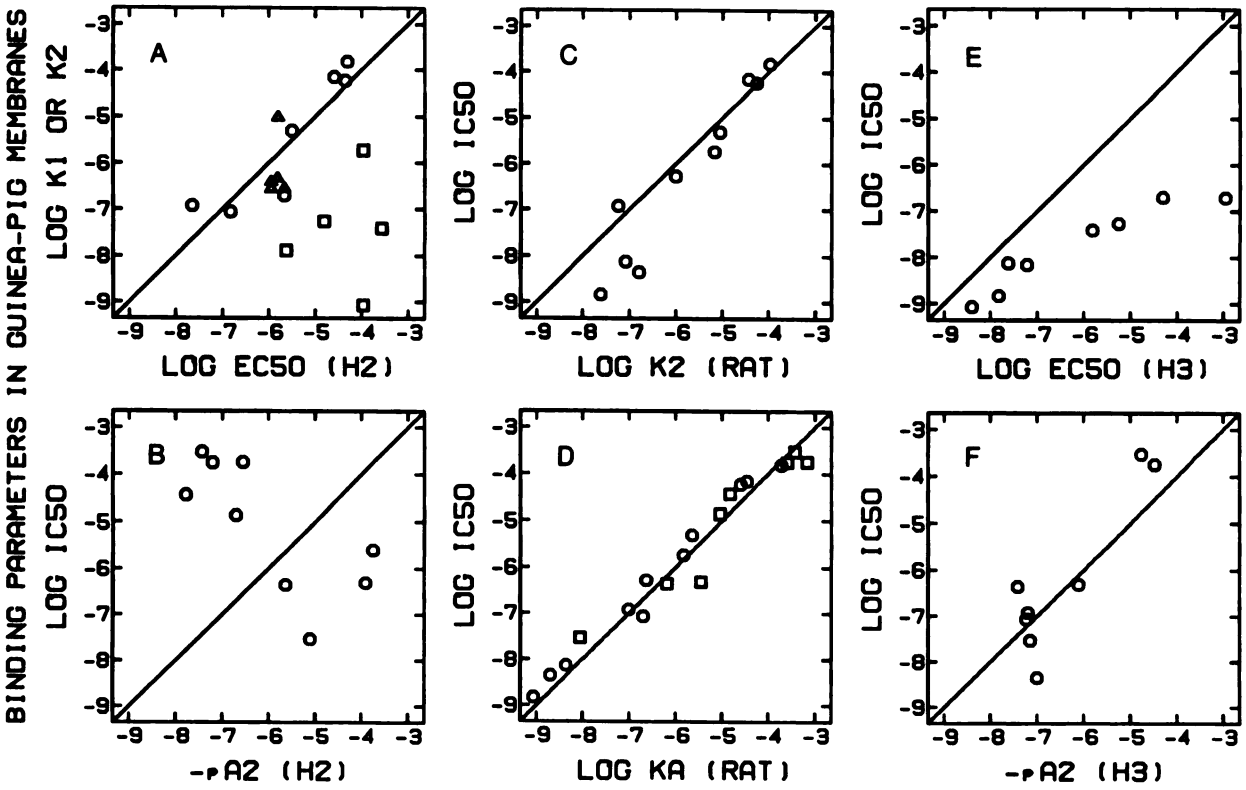

Fig. 6. Comparison of pharmacological specificity for binding and response in various preparations. *Values plotted on the ordinate in each panel* represent inhibitory potency with respect to the binding of [^{3}H]histamine to membranes from guinea pig cortex; *values plotted on the abscissa* relate either to binding in preparations from rat cerebral cortex or to response, as described below. A, Mean values of log K_1 (○) or log K_2 (△) for the binding of H_2 agonists (Table 3) are compared with values of log EC_{50} for the response of H_2 receptors in guinea pig right atrium ($r = 0.851$, $p = 0.00045$). Five compounds were omitted from the correlation (□), (*R*)-α-methylhistamine, nordimaprit, (*S*)-(+)-*N*$^\omega$-methyl-α-chloromethylhistamine, and imidazolylpropylguanidine. B, Mean values of log IC_{50} for the binding of H_2 antagonists (Table 3) are compared with values of pA_2 for the functional blockade of H_2 receptors ($r = -0.729$, $p = 0.026$). C, Mean values of log IC_{50} for the binding of H_2 agonists (Table 3) are compared with values of log K_2 for binding to sites labeled by [^{3}H]histamine in membranes from rat cerebral cortex ($r = 0.959$, $p = 0.00001$) (7). D, Mean values of log IC_{50} for the binding of H_2 agonists (○) and antagonists (□) (Table 3) are compared with mean values of log K_{A_1} (eq. 4) or log IC_{50} (eq. 3, burimamide) for binding to sites labeled by 8–12 nM [^{3}H]histamine in a digitonin-solubilized preparation from rat cerebral cortex ($r = 0.981$, $p < 0.00001$) (41, 42). E, Mean values of log IC_{50} for the binding of H_3 agonists (Table 3) are compared with values of log EC_{50} for the response of H_3 receptors ($r = 0.945$, $p = 0.0021$). F, Mean values of log IC_{50} for the binding of H_3 antagonists (Table 3) are compared with values of pA_2 for the functional blockade of H_3 receptors ($r = 0.899$, $p = 0.0024$). In all panels, the *line* indicates numerical equivalence.

fail to exhibit an H_2 specificity with respect to antagonists, the intriguing inhibitory behavior of H_2 agonists, and other properties found previously with membranes (41). The solubilized material contains two classes of sites similar in affinity and capacity to those described in the present report, and the relative capacities estimated in terms of scheme 2 are controlled by guanyl nucleotides and magnesium (41, 42). Particularly striking is the excellent agreement among 19 H_2 agonists and antagonists between apparent affinity in the solubilized preparation and the corresponding values of IC_{50} listed in Table 2 ($r = 0.981, p < 0.00001$) (Fig. 6D). The correlation is marginally weaker if IC_{50} is replaced by either K_1 ($r = 0.854$, $p < 0.00001$) or K_2 ($r = 0.901$, $p < 0.00001$) for those compounds that reveal two classes of sites.

The foregoing considerations suggest that [^{3}H]histamine labels the same sites in membranes from guinea pig cortex and in digitonin-solubilized preparations from rat cortex. The latter appear to be derived from H_2 receptors labeled by [^{3}H]histamine in membranes from rat cortex (42). An element of H_2 specificity emerges from the binding of H_2 agonists to membranes from guinea pig cortex when the appropriate value of K_j is compared with the functional potency of the compound at H_2 receptors (Fig. 6A); moreover, the labeled sites appear to distinguish between H_2 agonists and antagonists on the basis of their preference for one state or the other (Fig. 5; see also Table 2 in Ref. 22). Taken together, the data suggest that [^{3}H]histamine labels the same sites in all three preparations. The native properties of H_2 receptors, as characterized in the previous studies on membranes of rat cortex (5–7), appear to be labile upon solubilization (42) or even under the conditions of the present investigation in the case of membranes from guinea pig. The labeled sites described here also resemble those described originally by Barbin *et al.* (3): in particular, nine H_2 agonists show excellent agreement between the values of log K_1 in Table 3 and the binding constants presented in the earlier report (3) ($r = 0.988$, $p < 0.00001$).

Status as H_3 receptors. The compounds listed in Table 3 include 12 H_3 agonists and 12 H_3 antagonists. Explicit estimates of H_3 functional potency (EC_{50} or pA_2) are available for eight of each, and a comparison of pharmacological specificity raises the possibility that the labeled sites are H_3 receptors. Agonists reveal a significant correlation between their inhibitory potencies in the binding assay (IC_{50}) and the corresponding potency for blockade of the potassium-evoked release of histamine ($r =$

0.953, $p = 0.00025$). Values are consistently higher for EC_{50} than IC_{50} (Fig. 6E), an arrangement that recalls the comparison with functional potency at H_2 receptors (Fig. 6A), but the relationship is not improved if IC_{50} is replaced by K_1 or K_2. H_3 antagonists show a significant correlation between log IC_{50} and the reported values of pA_2 ($r = 0.907$, $p = 0.0019$) (Fig. 6F), although significance depends upon two compounds that bind 100-fold less tightly than do the other six. The correlation is weaker with K_2 taken in place of IC_{50} ($r = 0.877$, $p = 0.0042$) and not significant with K_1 ($r = 0.682$, $p = 0.063$).

H_3 receptors reportedly are labeled by (R)-α-[^{3}H]methylhistamine in membranes from rat cortex (18) and by N^α-[^{3}H]methylhistamine in membranes from guinea pig brain (16), membranes from AtT-20 pituitary tumor cells (17), and sections of rat forebrain (15). Both radioligands have been found to label an apparently homogeneous population of sites under most conditions, although the former has revealed two classes of sites in the presence of calcium (18); the Hill coefficient reportedly is 0.48 for the inhibitory effect of burimamide (18) and indistinguishable from 1 for other ligands. Capacity has been estimated at 29–41 pmol/g of protein (16, 17) or 25 fmol/section (15); comparable or somewhat higher values have been reported for the sites of nanomolar affinity for [^{3}H]histamine (~100 pmol/g of protein in Table 2 and Refs. 3 and 5; 20 fmol/section in Ref. 15). As illustrated in Fig. 7, several agonists and antagonists show good agreement between the values of log IC_{50} listed in Table 3 and their reported affinities for the sites labeled by N^α-[^{3}H]methylhistamine or (R)-α-[^{3}H]methylhistamine. Similarities in the binding properties suggest that N^α-[^{3}H]methylhistamine, (R)-α-[^{3}H]methylhistamine, and [^{3}H]histamine all label the same sites in mammalian brain.

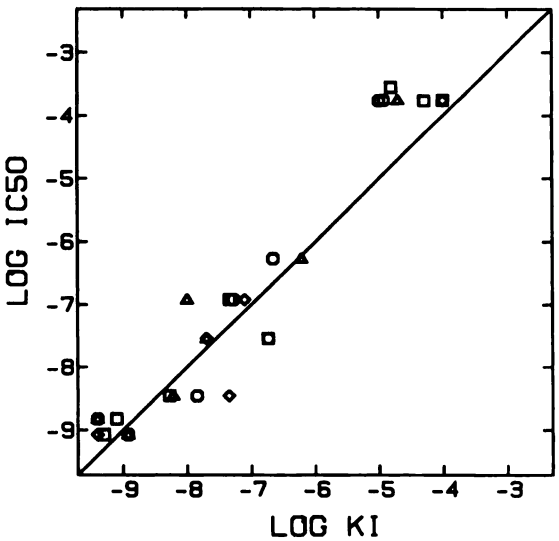

Fig. 7. Comparison of inhibitory potency (log IC_{50}) at the labeled sites in guinea pig cortex with apparent infinity (log K_i) at putative H_3 receptors. The values of log IC_{50} are taken from Table 3 and represent the concentration of agonist or antagonist that inhibits the specific binding of [^{3}H]histamine by 50%. The values of log K_i represent the reported affinity of each compound for the sites labeled by N^α-[^{3}H]methylhistamine in membranes from guinea pig cortex (○) (16), membranes from AtT-20 cells (△) (17), and sections of rat forebrain (◇) (15) and by (R)-α-[^{3}H]methylhistamine in membranes from rat cortex (□) (18). The correlation is significant for each set of data taken individually (○, $r = 0.952$, $p = 0.00027$; △, $r = 0.061$, $p = 0.00057$; X, $r = 0.957$, $p = 0.11$; □, $r = 0.974$, $p = 0.00039$) or for all of the data taken together ($r = 0.959$, $p < 0.00001$). The *line* indicates numerical equivalence.

Their pharmacological specificity and their probable identity as the target for N^α-[^{3}H]methylhistamine and (R)-α-[^{3}H]methylhistamine suggest that the sites labeled by [^{3}H]histamine in guinea pig cortex are H_3 receptors. Some caution may be in order, however, in view of the data illustrated in Fig. 6F; the correlation is wholly dependent on two compounds of relatively weak potency in both assays. Also, overall agreement between inhibitory potency in the binding assay and H_3 functional potency is poorer with the present data than with those presented elsewhere (16, 18); the difference arises primarily from compounds not listed in previous reports.

Possible basis of pharmacological uncertainty. The enigmatic behavior of the sites labeled by [^{3}H]histamine cannot be dismissed in terms of simple models or assigned vaguely to the potential complexity of native membranes. Previous authors have pointed out the potentially misleading effects of endogenous nucleotides on the properties of G protein-linked receptors (e.g., Ref. 43). Membranes used in the present investigation therefore were washed extensively in buffer containing EDTA, because guanyl nucleotides are known to dissociate rapidly at low concentrations of magnesium (e.g., Ref. 44). Experience with cardiac muscarinic receptors suggests that the washing procedure is effective in removing endogenous ligands without untoward effects on the binding properties or on the inhibition of GTP-stimulated adenylate cyclase (45). Similarly, the stimulation of adenylate cyclase via H_2 receptors was found to be comparable in washed and unwashed membranes from guinea pig cerebral cortex.[1]

Evidence for both H_2 and H_3 specificity raises the possibility that both receptors are labeled under the conditions of the binding assays; also, the probe might label subtypes of one receptor or the other, as suggested by Arrang *et al.* (18). A clear and recognizable specificity then is expected to emerge from a portion of the binding curve; moreover, the corresponding amplitude ought to be the same for all ligands, because the concentration of [^{3}H]histamine was the same throughout. In contrast, all but one of the relationships illustrated in Fig. 6 represent 100% of inhibitable binding, and the correlation generally is poorer when IC_{50} is replaced by K_1 or K_2 from eq. 3; in the exception (Fig. 6A), the fraction of labeled sites corresponding to the relevant parameter K_j varies from 9.7% to 100%. These considerations and the inadequacy of scheme 2 suggest that uncertainty over the identity of the labeled sites cannot be attributed solely to a mixture of H_2 and H_3 receptors. A yet unrecognized receptor or histaminergic subtype may be involved, or the binding properties may be determined in part by the conditions of the assays; for example, G protein-linked receptors are known to be modulated by monovalent cations (e.g., Ref. 46). Also, definition of pharmacological specificity in terms of scheme 2 may be naive.

For agonists at G protein-linked receptors, neither binding *per se* nor the relationship between binding and response is understood in mechanistic terms (47, 48). That uncertainty complicates the interpretation of any comparison between affinity as measured in binding assays and functional potency with respect to the response. Comparisons involving antago-

[1] W. G. Sinkins and J. W. Wells, unpublished observations. The activity of adenylate cyclase was measured as described previously (45). With preparations of previously frozen tissue, the production of [^{32}P]cAMP measured in the presence of 0.1 mM histamine and inhibitable by 10 mM cimetidine was 95 pmol/min/g of protein before and after washing; with fresh tissue, washing increased the response from 93 to 124 pmol/min/g of protein.

nists generally are more straightforward, because most systems appear to be organized such that those ligands are less sensitive to whatever phenomenon accounts for the multiple states reflected in the binding of agonists. The results described here and in the accompanying paper suggest that the receptors identified in guinea pig cortex behave differently; that is, the complexity of the system also is reflected in the binding of antagonists, as indicated by Hill coefficients markedly less than 1. Antagonists therefore may suffer from the same problems as agonists with respect to the relationship between binding and response. Furthermore, the data are manifestly inconsistent with the notion of distinct and independent sites (scheme 2); rather, one equivalent of ligand appears to affect the binding of another in a noncompetitive and possibly cooperative manner. Because scheme 2 is mechanistically inappropriate, the physical significance of the parameters derived from eq. 4 is unclear. It follows that the lack of a satisfactory correlation among antagonists between apparent affinity on the one hand and either H_2 or H_3 functional potency on the other may be misleading with regard to the identity of the labeled sites.

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