PURIFICATION OF HISTAMINE RECEPTOR (IV)
SPECIFICITY OF BINDING OF VARIOUS DRUGS TO THE HISTAMINE
RECEPTOR-RICH FRACTION AND TO SOLUBILIZED BINDING SITES

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Abstract—Studies were made on tritiated histamine binding to the receptor-rich mem-
brane fraction and solubilized sites and its displacement by various drugs. H1-Agonists
and antagonists displaced histamine most effectively. A H2-agonist and atropine
were less effective and propranolol, phentolamine and imidazole acetic acid had little
effect. The solubilized binding sites showed the same specificity of binding as the
membrane fraction. Membrane fragments had two binding constants, whereas
solubilized sites had only one. Solubilized sites bound similar amounts of histamine
and dibenamine: the latter was applied to intact tissue under conditions which would
presumably cause specific binding to histamine receptors. These binding character-
istics show that the method used was adequate for purification of histamine receptors
from smooth muscle of cat small intestine.

We have already succeeded in isolating a plasma membrane fraction rich in histamine
receptors, from the longitudinal smooth muscle of cat small intestine, after labeling the
tissue with radioactive dibenamine utilizing a protection technique (1–3). Solubilization
of the labeled binding sites has been described briefly (2). Tissue fractions were separated
after treating the tissue with drugs. These drugs acted through pharmacological receptors
localized on the intact plasma membrane (3). The binding specificities of the fragmented
membrane fraction are criteria for the presence of active membrane bound receptors in the
fraction. Accordingly, we examined the direct binding of drugs to fragmented membranes
isolated from intact tissues. We also investigated the binding specificities of solubilized
sites. Studies on the binding of drugs to β-adrenergic receptors (4–6) and other receptors
(7–9) in vitro have been reported. This paper is concerned with the binding of tritiated
histamine to the receptor rich membrane fraction and to the solubilized sites.

MATERIALS AND METHODS

The receptor rich membrane fraction was prepared as described previously (3). For
preparation of dibenamine-treated receptor rich fraction, the muscle layer was incubated
with dibenamine (4×10^-6 M) alone for 20 min in Tyrode solution (pH 7.6–7.8, at 37°C).
A fraction in which histamine receptors were protected from dibenamine was prepared from
muscles incubated with dibenamine (4×10^-6 M) in the presence of diphenhydramine (1×10^-6

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The fractions were washed with Locke-Ringer solution and then suspended in 15 mM Tris-maleate buffer (pH 7.4) containing 1 mM EDTA-Mg and 1% (v/v) Triton X-100 (Sigma) at a final concentration of 1 μl of 100% Triton/mg protein and gently homogenized with 5 strokes by a Teflon homogenizer. The homogenate was stirred for 30 min at 4°C and then centrifuged at 105,000 x g for 1 hr. Triton was removed from the supernatant with 1 g/ml of Bio-Beads SM-2 (Bio-Rad Lab., Richmond, Calif.) and the absorbance was measured at 275 nm (10). The solution was then passed through a Millipore filter (0.45 μm pore size) and the filtrate was assayed for binding activity.

For assay of binding to the receptor rich membrane fraction, 1 ml of the suspension of the fraction (0.1-0.2 μg protein) was incubated with tritiated histamine (10⁻⁸ M, 62,000 dpm/ml) in Locke-Ringer solution for 10 min. The test compounds were added to the incubation mixture as indicated. Binding was estimated by passing the mixture through Millipore filters (HA 0.45 μm pore size) and counting the radioactivity on the filter as described previously (11).

For assay of ³H-histamine binding to solubilized sites, samples of solubilized materials (10-20 μg protein/ml) was dialysed against ³H-histamine (10⁻⁹ M) for 2 hr by the microdialysis method of Anraku (12). Protein was measured by the microbiuret method (13). Binding experiments were carried out at 20°C.

RESULTS

³H-Histamine bound rapidly and reversibly to sites in muscle membrane fragments. On addition of excess unlabeled histamine (10⁻⁹ M) after equilibrium had been achieved, 80% of the bound ³H-histamine was dissociated within 3 min. Scatchard plots (14) of the results are shown in Fig. 1. The data were not consistent with a model containing a single homogeneous order of sites. However, the data were consistent with the presence of two orders of sites, one of K₁ = 2.1 x 10⁸ M⁻¹ and the other of K₂ = 1.3 x 10⁴ M⁻¹. The binding capacities of the two corresponded to about 2 n moles/mg protein for the higher order site and 17 n moles/mg protein for the lower order site. Whether or not the data fit models with a larger number of sites was not determined. The binding activities of a dibenamine-
treated preparation and a protected preparation were compared (Fig. 2). The former preparation did not have these high order sites. The protected preparation contained both high and low order sites; the constant \( K_1 \) of high order sites was similar to that in the preparation from intact muscles and the low order sites of \( K_2 = 1.6 \times 10^4 \) M\(^{-1} \) were a little

![Fig. 2A](image1)

![Fig. 2B](image2)

**Fig. 2.** Scatchard plots of \(^3\)H-histamine binding to the receptor-rich fraction prepared from dibenamine-treated muscles. Conditions were as for Fig. 1. A; Fraction from the muscle treated with dibenamine alone \( (4 \times 10^{-6} \) M, for 20 min). Histamine receptors were blocked with dibenamine. B; Fraction from the muscle protected from dibenamine with diphenhydramine \( (1 \times 10^{-6} \) M). Dibenamine did not contact histamine receptors.

![Fig. 3A](image3)
FIG. 3. Inhibition of $^3$H-histamine binding to the receptor-rich fraction by various drugs. Values are means of triplicate determinations. Protein concentration was 0.1 mg/ml. A: a, Chlorpheniramine; b, Diphenhydramine; c, Histamine; d, Atropine; e, Phentolamine; f, Propranolol; g, Physostigmine. B: a, Histamine; b, 2-Methyl-histamine; c, Betazole; d, 4-Methyl-histamine; e, Imidazole acetic acid (Phenytoin, Isoproterenol and Carbachol).

FIG. 4. Inhibition of $^3$H-histamine binding to solubilized sites by various drugs. Values are means of triplicate determinations. Protein concentration was 10 $\mu$g/ml. a, Diphenhydramine; b, Histamine; c, 2-Methyl-histamine; d, 4-Methyl-histamine; e, Atropine; f, Propranolol; g, Imidazole acetic acid. 0.1 ml of the solubilized material was dialysed against 2.0 ml of 15 mM Tris-maleate buffer (pH 7.4) containing 3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM KCl, $10^{-3}$ M $^3$H-histamine and various concentrations of unlabeled drugs. The radioactivity of the dialysate was counted in a liquid scintillation counter.

FIG. 5. Scatchard plot of $^3$H-histamine binding to solubilized sites. $5 \times 10^{-9}$ to $3 \times 10^{-4}$ M histamine was used. Protein concentration was 20 $\mu$g/ml. Values are means of triplicate determinations. 0.1 ml of the solubilized material was dialysed against 2.0 ml of 15 mM Tris-maleate buffer (pH 7.4) containing 3 mM MgCl$_2$, 1.2 mM CaCl$_2$, 10 mM KCl and various concentrations of $^3$H-histamine.
higher than that in the intact preparation.

To investigate the specificity of binding, the inhibitory effects of various drugs were examined and the inhibition curves obtained are depicted in Fig. 3. Similar results were obtained on the binding characteristics of solubilized sites (Fig. 4). Fig. 5 shows a Scatchard plot of data obtained with a solubilized preparation. The linear plot indicates the presence of a single order of saturable binding sites and the slope gives a constant of \(1.1 \times 10^5\) M\(^{-1}\). The results show that the sites were 4 n moles/mg protein.

**DISCUSSION**

A receptor-rich fraction and solubilized binding sites from smooth muscles of cat small intestine showed many of the binding properties of physiological histamine receptors in the small intestine (1). With these preparations, histamine, diphenhydramine, chlorpheniramine, 2-methyl-histamine—a relatively selective H\(_1\) receptor agonist (15)—, and betazole were the most effective in displacing histamine. Imidazole acetic acid—a metabolite of histamine—, physostigmine, phenylephrine, isoproterenol and carbachol were not effective. Atropine, 4-methyl-histamine—a H\(_2\)-receptor agonist (15)—, phentolamine and propranolol were effective at higher concentrations than H\(_1\)-receptor agonists and antagonists. Phentolamine has a histamine-like action (16) and propranolol binds nonspecifically to the membranes (17). Thus the specificity of binding of preparations *in vitro* is less than that of evoking physiological responses in intact tissue preparations. Similar results were obtained with fractions from protected muscle in displacement experiments. The concentration of other drugs required to displace histamine was higher than those of H\(_1\)-receptor agonists and antagonists. The strong effects of H\(_1\)-type histaminergic drugs in displacement experiments are good evidence that the binding sites are indeed on the membrane fragments and in the solubilized form as histamine receptive sites.

Scatchard plots of results obtained with dibenamine-treated and protected fractions suggested that dibenamine had made contact with the receptor in dibenamine-treated preparation but also that sites with low affinity for histamine might still be present. The receptor-rich fraction labeled with dibenamine ‘specifically’ bound 1.2 n moles of \(^3\)H-dibenamine/mg protein as receptor label and the solubilized sites bound 2.2 n moles (2). Scatchard plots indicated that 2 n moles of histamine/mg protein were bound to higher order sites in the membrane fraction and 4 n moles to solubilized sites. For more detailed experiments on the solubilized sites, the detergent must be completely removed and such is now being attempted by replacing the detergent with membrane lipid mixtures (18).

Takagi et al. (19) reported that Ca\(^{2+}\) and Mg\(^{2+}\) ions are essential for the combination of agonists with their receptors such as cholinergic, histaminergic and \(\alpha\)-adrenergic. Takayanagi et al. (20) confirmed this by measuring the binding of \(^3\)H-histamine to the histamine receptor-rich fraction of cat small intestinal smooth muscle (2) in the presence and absence of Ca\(^{2+}\) and Mg\(^{2+}\) ions and showing that cations increased histamine binding. The number of the receptor was calculated assuming that increase in histamine binding induced by cations was due to binding to histamine receptors (21): the increase in binding of histamine
was found to be 20 p moles/mg protein of the receptor-rich fraction. The binding was investigated using 10^{-8} M histamine (20), as in the displacement experiments reported here. In contraction of intact muscles, histamine was effective at a concentration above 10^{-7} M (1, 19). With the low concentration used in this work, only part of the receptors may be bound to histamine but this low concentration was used in studies on the specific binding of histamine to the receptors, because higher concentrations would increase non-specific bindings. The similarities in the amounts of binding of dibenamine and histamine or of drugs in vivo and in vitro indicate that the receptors on the fragmented membrane were not damaged. However, the value calculated for the number of sites bound to drugs was unexpectedly high, although the calculation necessarily involved several assumptions (22). Only part of the total amount of bound agonists may be related to physiological events.

The binding characteristics of atropine, 4-methyl-histamine, phentolamine and propranolol indicate that even the sites which bind to histamine with high affinity, may include sites other than histamine receptor. When added at a high concentration, these drugs may act on H1-receptors but they may also bind to other sites. It has been reported (23) that pharmacological agents with protective actions combine with other sites besides receptors. Radioactive dibenamine and histamine made contact not only with the receptors but also with other non-specific sites, even in the preparation protected from the action of non-radioactive dibenamine. Consequently, the number of sites bound to drugs was unexpectedly high. For covalent labeling of membrane (24) further studies are required on the specificities of alkylating agents and photoaffinity labeling agents (25-27).

The specificities of protective agents are equally important. In further purification of the sites, we are now trying to use two or more protective drugs. Preliminary examination showed that treatment with several drugs reduced the amount of 'specifically' bound dibenamine by at least half.

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