Purification of Histamine Receptor (VI) 
An Improved Double Labeling Method with "Double Protection"

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Abstract—Studies were done on the specific labeling of the histaminergic H1-receptor of the longitudinal smooth muscle of cat small intestine. A procedure involving 'double protection' combined with the double labeling technique was developed. The first protection was the usual with a protective antihistamine, promethazine, and the second was cross protection of non-specific sites with non-histaminergic drugs, thiophrine and atropine. Muscle tissue protected with promethazine against non-radioactive dibenamine was treated with 3H-dibenamine in the presence of these second protectors. The second protectors covered non-receptor sites which had been protected from non-radioactive dibenamine with promethazine. The dose-response curves were carefully checked in each experiment to confirm that the second protectors did not interfere with the specific coverage provided by the first protector. Finally 14C-dibenamine was applied to measure non-specific binding after which the labeled muscles were fractionated and the radioactivity was counted. The specificity of labeling achieved in the receptor-rich fraction by this method is discussed.

Using radioactive dibenamine as a marker, Uchida and Takagi demonstrated that in the longitudinal smooth muscle of cat small intestine, H1-histaminergic receptors were located on the plasma membrane (1-6), where active extrusion of calcium ions (6) and H1-receptor-mediated calcium ion movements (7) were observed. The receptive sites were solubilized by a detergent (2, 4) and from results on displacement of 3H-histamine with various drugs (4) i.e. radio-receptor assay—, it was concluded that the receptor sites have similar binding characteristics to intact muscle strips even after subcellular fractionation and solubilization. Analysis by Scatchard plots (8), however, indicated that the binding sites of histamine included sites other than active receptors. After studying the kinetics of binding of atropine and methylatropine, Paton and Rang (9) reported that the protective drugs combine with other sites in addition to the pharmacological receptors. Only a small fraction of the bound drugs seems to be directly concerned with the specific action in intact muscle strips and also in vitro. It therefore seems necessary to label the receptor sites more specifically in order to pursue the specific site of drug-receptor binding. So-called non-specific sites, which bind to histaminergic drugs and/or dibenamine without evoking physiological or pharmacological events, should not be labeled. Dibenamine irreversibly blocked histaminergic H1-receptors even after repeated washing of the tissue for more than 8 hr (1, 10). Dibenamine bound to the tissue by alkylating nucleophilic sites (1). Dibenamine

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can discriminate histamine receptors pharmacologically from other receptors on smooth muscle cells when a protection technique (9, 11) is utilized. For protection, receptors are covered with an agonist or antagonist which can be removed by wash out. One cause of chemically non-specific labeling is the non-specificity of the alkylating agent in a chemical sense and the other is the non-specificity of the protectors to prevent the alkylation of the receptor sites. For the first item, a more specific alkylating agent for histamine receptors has been synthesized by our group (12). Therefore, in this work special concern was paid to the specificity of protection, in a chemical sense. Two-step-application of two kinds of protectors—'double protection'—was developed in combination with a modification of double labeling procedure reported previously (5). The double labeling procedure decreased the influence of difference in sensitivities of muscle strips to drugs, because only a single muscle is required for comparison between specific and non-specific labeling. For successful 'double protection', the first step was the usual with a protective antihistamine and the second was cross protection with non-histaminergic drugs of non-H\textsubscript{1}-receptor sites which had been protected with the first protector 'non-specifically'. In this paper, an adequate procedure is reported for labeling H\textsubscript{1}-receptors in smooth muscle of cat small intestine.

MATERIALS AND METHODS

Pharmacological tests of drugs

The longitudinal smooth muscle of cat small intestine, obtained as described by Takanayanagi et al. (13), was cut into a long strip (in situ, approx. 25 cm long) and suspended in a long organ bath (50 cm long \times 5 cm diameter, made from a chromatographic column with a circulating system) filled with Tyrode solution kept at 22°C, with aeration. The contraction of the strip was reduced through a lever and recorded on an ink writing kymograph. The antihistaminic action or protective effect of the drug was tested and contraction heights were compared to the maximal response to acetylcholine whose action was not affected by dibenamine under the present conditions (1). Muscle tissue highly sensitive to dibenamine was collected and from the results with non-radioactive dibenamine as shown in Figs. 1 and 2, a suitable labeling procedure was concluded to be that as shown in Fig. 3 (right).

The effects of drugs on the muscle were tested in each experiment with radioactive dibenamines which were equi-active to non-radioactive dibenamine (AKAO, M., Doctor Thesis, Univ. of Tokyo, 1963). Representative curves are shown in Fig. 3 (left) and muscle tissue having different drug-sensitivities, particularly to dibenamine, was discarded.

To determine radiochemical protective effects of the two cross protectors, i.e. thioridazine and atropine, muscle strips were treated by the same procedure, except that these two drugs were used and the resulting radioactivities were counted.

Preparation of the receptor fraction

The fraction rich in histamine receptors was obtained from tissues labeled according to the method shown in Fig. 3 as reported previously (3). The fraction was resuspended in 0.6 M KCl (in 20 mM Tris-maleate buffer, adjusted to pH 7.2) for 2 hr under gentle stirring to dissolve contaminated actomyosin and then centrifuged at 50,000 \times g for 40 min. The
resulting pellet was resuspended in 20 mM Tris-maleate buffer (pH 7.4) and used as the membrane fraction. The membrane fraction was filtered through a Sephadex G-200 column (2). Elution was performed with 50 mM Tris-maleate (pH 7.4) containing 0.5 M NaCl at 4°C. The second peak between blue dextran and apoferritin (MW. 466,000) was collected and concentrated with Amicon membrane filters (CF 50A). Triton x-100 was added to the concentrated fraction to make a final concentration of 1% (v/v) with gentle homogenization in a Dounce-type homogenizer (2). The suspension was allowed to stand for 30 min at 4°C and then centrifuged at 75,000 × g for 40 min. The suspension was applied to a Sephadex G-100 column and eluted with 50 mM Tris-maleate (pH 7.2) containing 0.1% Triton x-100. The second peak between γ-globulin (MW. 166,000) and transferrin (MW. 77,000) was collected and concentrated with Amicon filters (CF 25).

The tissues used as controls to the longitudinal smooth muscle of cat small intestine were the circular muscle of cat small intestine and the longitudinal and circular layers of small intestinal smooth muscle of the dog. These tissues were treated in the same manner as the longitudinal smooth muscle of cat small intestine and the membrane fractions were prepared as described above.

Radioactivities were counted after oxidizing the sample automatically in a Model 306 Tri-carb oxidizer (Packard Instrument Co.). Protein was determined by the microbiuret method (14).

Materials

Tritiated dibenamine hydrochloride (58.5 Ci/mole) was synthesized by Akao as reported by Takagi and Uchida (1). 14C-Labeled dibenamine (9.5 Ci/mole) was prepared by Sumitomo Nuclear Co. Ltd. (1). Other drugs were as follows; acetylcholine chloride (Dai-Ichi), histamine dihydrochloride (Wako), thioridazine hydrochloride (Sandoz), promethazine hydrochloride (Wyeth), atropine sulphate (Merck) and dibenamine hydrochloride (recrystallized, Tokyo Kasei). For standards of gel filtration; blue dextran (Pharmacia), apoferritin (horse spleen, Sigma), γ-globulin (bovine, Wako) and transferrin (human, Sigma) were used.

RESULTS

Promethazine (3 × 10⁻⁷ M) antagonized the action of histamine in the longitudinal smooth muscle of cat small intestine but thioridazine (1 × 10⁻⁶ M) and atropine (3 × 10⁻⁷ M) had little effect on the action. As shown in Fig. 1, a mixture of thioridazine and atropine shifted the dose-response curve for histamine slightly to the right but such was significant. When these drugs were applied concomitantly to protect the receptors from the effect of dibenamine (1 × 10⁻⁶ M, for 20 min) the dose-response curve shifted significantly to the right and the maximal contraction height decreased by 45%. The action of dibenamine was not significantly different in the presence and absence of the two drugs. Thus the histamine receptors were blocked with dibenamine even in the presence of the two drugs. However, the radioactivity bound in the absence of the two cross protectors was 85 ± 5 p moles/mg protein of the membrane fraction (N=5) whereas that bound in their presence was only
FIG. 1. Dose-response curves for histamine in the presence of thioridazine and atropine. A piece of longitudinal smooth muscle of cat small intestine was suspended in an organ bath filled with Tyrode solution kept at 22°C with aeration. Cumulative responses were recorded isotonically and were expressed with percentages relative to the maximum contraction height induced by acetylcholine. *, control; A, in the presence of thioridazine (1 x 10⁻⁶ M) and atropine (3 x 10⁻⁷ M); 2 hr after treatment with dibenamine (1 x 10⁻⁵ M, for 20 min) in the presence of thioridazine (1 x 10⁻¹ M) and atropine (3 x 10⁻⁷ M): [, 2 hr after treatment with dibenamine (1 x 10⁻⁶ M, for 20 min) in the absence of thioridazine and atropine. Values are averages of those in six preparations.

FIG. 2. Dose-response curves for histamine in the presence of promethazine. •, control; after incubation for 5 hr: △, 3 hr after treatment with dibenamine (8 x 10⁻⁶ M, for 20 min) in the presence of promethazine (1 x 10⁻⁶ M); A, 4 hr after treatment with dibenamine (4 x 10⁻⁶ M, for 20 min) in the presence of promethazine (3 x 10⁻⁷ M); [, 2 hr after treatment with dibenamine (1 x 10⁻⁶ M, for 20 min) followed by 2 hr washing: (e'), 4 hr after treatment with dibenamine (1 x 10⁻⁶ M, for 20 min) followed by 2 hr washing. Values are averages of those in five preparations. Other notes as in Fig. 1.

40 ± 3 p moles (N = 5). Thus the cross protectors effectively protected other sites or non-specific sites which could bind to dibenamine without affecting the histamine receptors. When dibenamine (4 x 10⁻⁶ M, for 20 min) was applied in the presence of promethazine (3 x 10⁻⁷ M), the curve shifted slightly to the right and the maximum contraction height decreased by 5%. In the presence of promethazine (1 x 10⁻⁶ M), dibenamine (8 x 10⁻⁶ M, for 20 min) did not shift the curve, as shown in Fig. 2. Therefore under the latter conditions, protection of histamine receptors was not complete and 'q value' calculated as described by Furchgott (15) was approx. 0.5.

When dibenamine (1 x 10⁻⁶ M, for 20 min) was again applied in the presence of the two cross protectors to the muscle which had been protected with promethazine (3 x 10⁻⁷ M) as described above, the dose-response curve shifted significantly to the right. The maximum contraction height was reduced to 45%. Histamine receptors were blocked by the second application of dibenamine and the 'q value' after the second treatment was approx. 0.02.

On the basis of these results, a labeling procedure was designed for use in combination
with the double labeling procedure (Fig. 3, For details see DISCUSSION). The radioactivities found in subcellular fractions and solubilized materials of the longitudinal muscle of cat small intestine are listed in Table 1. The distribution of labeled receptors is indicated by tritium-label and the ratio of tritium to $^{14}$C is a criterion of purification. Tritiated dibenamine was concentrated in the membrane fraction and the ratio increased with increase

![Fig. 3. Flow sheet of labeling of histaminergic H₁-receptors in longitudinal smooth muscle of cat small intestine. The labeling procedure on the right was determined from the results in Figs. 1 and 2. The dose-response curves on the left were obtained with cold, tritiated or $^{14}$C-labeled dibenamine at the indicated step. The number of treatments (1, 2 and 3) corresponds to those in Fig. 4. Values are averages of those in five preparations. For an explanation of the cross protection with thioridazine and atropine, see TEXT and Fig. 4.]

**TABLE 1.** Radioactivities in the longitudinal smooth muscle of cat small intestine and subcellular fractions of the muscle

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$^{3}$H</th>
<th>$^{14}$C</th>
<th>$^{3}$H/$^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>5</td>
<td>7.12</td>
<td>150±20</td>
<td>0.05</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>5</td>
<td>40±3</td>
<td>600±50</td>
<td>0.07</td>
</tr>
<tr>
<td>Sephadex G-200 eluate</td>
<td>5</td>
<td>100±8</td>
<td>430±40</td>
<td>0.23</td>
</tr>
<tr>
<td>Solubilized fraction</td>
<td>5</td>
<td>270±24</td>
<td>340±30</td>
<td>0.79</td>
</tr>
<tr>
<td>Sephadex G-100 eluate</td>
<td>3</td>
<td>960±80</td>
<td>180±10</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Values are expressed as $p$ moles of dibenamine per mg protein (mean±S.E.). n: number of preparations.

**TABLE 2.** Radioactivities in Sephadex G-200 eluates of membrane fractions from intestinal microsomes

<table>
<thead>
<tr>
<th></th>
<th>$^{3}$H</th>
<th>$^{14}$C</th>
<th>$^{3}$H/$^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal</td>
<td>100±8</td>
<td>430±40</td>
<td>0.23</td>
</tr>
<tr>
<td>Circular</td>
<td>20±4</td>
<td>400±50</td>
<td>0.06</td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longitudinal</td>
<td>25±3</td>
<td>380±40</td>
<td>0.07</td>
</tr>
<tr>
<td>Circular</td>
<td>20±4</td>
<td>400±50</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Values are expressed as $p$ moles of dibenamine per mg protein (mean±S.E.). Four preparations.
in purification of the membrane fraction and solubilized materials. The amount of tritiated dibenamine in other muscles was much less than in the longitudinal muscle of cat small intestine, as shown in Table 2: The amount in the membrane fractions from muscles which did not respond to histamine (up to $3 \times 10^{-4} \text{ M}$) was approximately $1/4$ of that in the longitudinal muscle of the cat.

**DISCUSSION**

Since the possibility of labeling receptors with $\beta$-halogenoethylamines was suggested \cite{10, 11}, attempts have been made to label $\alpha$-adrenergic receptors using dibenamine \cite{16-20}, phenoxybenzamine \cite{21, 22} and SY-28 \cite{23-27}. Dibenamine was also used to label muscarinic receptors \cite{28-30} and histaminergic $H_1$-receptors \cite{1-6}. Recently, attempts were made to label muscarinic receptors with benzylcholine mustard and its analog \cite{31-35}. In most of these studies the receptors were completely protected. In the present study, some of them were labeled. As shown schematically in Fig. 4, a high dose of non-radioactive dibenamine was first applied in the presence of a protective antihistamine, under the condition where the protection was not complete. After washing out the protector, $^3\text{H}$-dibenamine was applied at a lower concentration under the condition where histamine receptors were blocked. On this second treatment with dibenamine in combination with drugs which would provide a cross protection, non-specific binding of this second dibenamine ($^3\text{H}$) could be greatly reduced. The cross protectors covered non-specific sites which were to bind with dibenamine but which had been covered by the first protector.

The second protector should resemble the first in structure, but should not act on $H_1$-receptors in the concentration used. The author chose promethazine as the first protector and thioridazine as one of the second protectors; both these drugs are phenothiazines. The former is a potent antihistamine and the latter has little antihistaminic action \cite{36}. Atropine was also used as it has antihistaminic action when applied in high concentrations. At the concentration used in this experiment, atropine had no antihistaminic action and few histaminergic sites were protected pharmacologically by the two cross protectors against dibenamine. However, radiochemical protection was observed. These cross protectors protected non-specific sites effectively, because their pharmacological action were carefully checked in each experiment herein in order to avoid a synergistic effect of their antihistaminic action, which in turn would protect histamine receptors pharmacologically. Thus $^3\text{H}$-dibenamine applied in the presence of these cross protectors to the muscle which had been protected with promethazine is the marker of the histamine receptor. $^1\text{C}$-Dibenamine was then applied for a third time in the presence of promethazine ($1 \times 10^{-8} \text{ M}$). This application of dibenamine served as an indicator of non-specific sites, as the receptor remaining in an active form after the second application of dibenamine ($^3\text{H}$) were protected with promethazine at a sufficiently high dose and binding to $^1\text{C}$-dibenamine was negligible.

The crucial points in the present method were the incomplete protection of receptors before labeling them, labeling of the receptor in the presence of cross protector and the application of $^1\text{C}$-dibenamine after labeling receptors with $^3\text{H}$-dibenamine. The first and
FIG. 4. Schematic drawing of labeling of H₁-receptors by means of 'double protection' combined with double labeling procedure. The numbers at the top of the figure (1, 2 and 3) correspond to those of treatments shown in Fig. 3. The areas of the receptors and the non-specific sites are schematically depicted on the right. The horizontal bars under the numbers of treatments represent the binding of dibenamine, whereas the areas indicated by vertical arrows are those covered with the protector(s). As the first treatment (1), a high dose of non-radioactive dibenamine was applied in the presence of an antihistaminic phenothiazine, promethazine, which covered the receptors. Under this condition dibenamine was mainly bound to non-specific sites but a part of the receptors was bound to this dibenamine. Promethazine, on the contrary, covered a part of non-specific sites in the first treatment. By the second treatment (2), a greater part of this non-specific sites which had been covered by the promethazine, was again covered by a non-histaminergic phenothiazine, thioridazine, and by atropine. In the presence of these cross protectors, the second dibenamine (³H) was applied in a lower concentration and a considerable amount of the receptor was bound to this dibenamine. The quantity of ³H-dibenamine bound to non-specific sites was small compared to that bound to the receptors. Thus ³H-dibenamine should mark the receptor sites. Finally in the third treatment (3), the highest concentration of dibenamine (¹⁴C) was applied in the presence of a high dose of promethazine, which covered the area including remaining receptors still in active form after the second treatment. ¹⁴C-Dibenamine should, therefore, act on non-specific sites. This application of dibenamine denoted non-specific sites. The presence of cross protectors in the second treatment with a low dose of dibenamine (³H) provided a satisfactory specificity in labeling H₁-receptors highly sensitive to dibenamine.

second points resulted in specific binding of ³H-dibenamine to the receptors and the third in binding of ¹⁴C-dibenamine to the other sites. The membrane fraction was rich in 5'-nucleotidase, which is a general marker of plasma membrane. The membrane fraction also showed high activities of Ca²⁺-activated ATPase and Na⁺-, K⁺-activated ATPase although these activations were tested in 20 mM histidine buffer (pH 7.4) under low Mg²⁺ concentration which showed approx. half of the maximal activation induced by Mg²⁺ alone. From the analysis of total lipids (30% of dry weight of the fraction), a high molar ratio of cholesterol to phospholipids (0.91) was obtained. Therefore Uchida and Takagi concluded that the membrane fraction was mainly composed of plasma membrane where H₁-receptors were enriched (3). The radioactivity of ³H-dibenamine found in the membrane fraction in
the present work was about one tenth of that obtained by the method used previously (1–3). This radioactivity was solubilized and concentrated by gel filtration. About 1 n mole of dibenamine was bound per mg protein of the solubilized materials. This value is much the same as that reported previously but the significance of the value is different, because the essential point is the extent of concentration of the label: in previous work, the label was concentrated 2-fold by solubilization whereas in this study it was concentrated 10-fold. In previous experiments, the label was probably contaminated with non-specific labeling, most of which is considered to be protected by the second protectors used in the present study. The radioactivity of 14C-dibenamine, the marker of non-specific sites, was also found in the membrane fraction but such decreased in more purified fraction: solubilized materials contained about 1/20 of the total 14C-dibenamine of the tissue.

Membrane fractions obtained from histamine-insensitive smooth muscles were scarcely labeled with 3H-dibenamine but their contents of 14C-dibenamine were similar to that of the longitudinal smooth muscle of cat small intestine. Thus it is concluded that most of the 3H-dibenamine was bound to the active histamine receptors present in the longitudinal muscle of cat small intestine but not in the histamine-insensitive smooth muscles tested in this study. By this method, it is possible to achieve more specific binding of dibenamine to the receptor or the peri receptor area (37) at least than that reported previously for histaminergic receptors (1–3) or other receptors (16–35).

The failure to achieve specific labeling of α-adrenergic receptors with β-halogenoethylamines (16–22, 25–27) was attributed to the fact that the irreversible blockade were not the result of alkylation of the α-adrenergic sites but rather the result of inhibition of ‘Ca sites’ or ‘Ca binding/mobilizing sites’ (38–40). Recently Ohmura et al. (41, 42) showed that the irreversible blockade of contraction to acetylcholine, K+ or Ba2+ was due to inhibition of ‘Ca sites’ by dibenamine. Unlike α-adrenergic and acetylcholine receptors or the sites of action for K+ and Ba2+, these workers showed that dibenamine alkylates histamine receptors per se to block receptor-histamine interaction. Therefore, dibenamine may be reconsidered a suitable drug for labeling H1-receptors. Dibenamine does not bind specifically to the receptor sites but using appropriate protectors it can be used to label the H1-receptor highly sensitive to dibenamine. The ‘double protection’ method effectively overcomes the limitation of the previously used methods. With a more specific irreversible blocker (12), it should be possible to obtain more detailed information on the H1-receptors.

Further, this double protection will be useful for other receptor labeling. For example, in muscarinic cholinergic receptor labeling with a benzylcholine mustard analog (31–35), (+) benzetimide with an anticholinergic action might be adequate as a first protector and the (−)-isomer without any anticholinergic action suitable as the second protector (43, 44).

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