PURIFICATION OF HISTAMINE RECEPTOR IN CAT SMALL INTESTINAL MUSCLE

I) SPECIFICITY OF LABELING HISTAMINE RECEPTOR WITH RADIOACTIVE DIBENAMINE

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The interaction of a drug with a specific site of tissue cells, i.e. drug receptor, is generally assumed for explaining the mode of action of some drugs, but very little is known on its substantial existence and further on its chemical natures. The best method available for solving this problem is specific alkylation of the receptor with a labeled β-haloethylamine in combination with an antagonist or agonist. Testing the parallelism between incorporated radioactivity and pharmacological phenomena, rather specific labeling of drug receptors can be expected. The point on which emphasis was laid in this study was to distinguish the pharmacological blocking effect of dibenamine from its chemical alkylating ability. All the amount of radioactivity found in the tissue is not limited to dibenamine concerned in the pharmacological blocking action.

During this study (1-4) several papers were published on specific labeling of α-adrenergic receptor in various tissues with labeled β-haloethylamines utilizing simultaneously an antagonist and/or an agonist (5-10).

In these reports, however, the correlation between the incorporated radioactivity and the pharmacological effect of a β-haloethylamine was not sufficiently confirmed.

To avoid these weak points following experimental designs were chosen.

1. Three kinds of pharmacologic protective drugs were used.
2. Three kinds of pharmacologically non-protective drugs were also employed in the same manner as the protective drugs.
3. Six kinds of tissues with different histamine sensitivities were treated in the same way as the cat small intestine.

These principles will testify the assumption that the receptor is specifically labeled with dibenamine.

MATERIALS AND METHODS

1. Materials

Tritiated dibenamine (³H-Dib) was synthesized by Akao (11). The specific radioactivity was 58.5 mCi/mMole. C¹4 labeled dibenamine (¹⁴C-Dib) was prepared by Sumi-
tomo Nuclear Co. Ltd. and the specific radioactivity was 9.5 mC/mMole. Purity of dibenamine synthesized was assayed by a thin layer chromatography, an infra-red spectrum and an elemental analysis. The thin layer was Kiesel gel G and the developing solvent was cyclohexane-benzene (1:1). As the result of these analyses, no significant difference was observed between labeled dibenamine and standard dibenamine.

2. Preparation of tissue strips

Cats weighing 2.0–3.5 kg were anesthetized with sodium pentobarbital 30 mg/kg and after blood depletion by cutting the carotid arteries organs were excised. Excised organs were immediately immersed into oxygenated Tyrode solution.

The small intestine was cut open longitudinally along the adipose tissue where the mesentery was attached with the small intestine. After blood vessels and the adipose tissue were removed, the mucous layer was peeled off from the intestine and successively the circular muscle layer was removed paying attention not to injure the longitudinal muscle layer. The tracheal muscle was freed from the cartilage and a heart muscle strip was prepared from a part of the left atrium. A left leaf of the liver, the diaphragm and rectus abdominis were also used. The above organs were freed from the adipose tissue and blood vessels as far as possible. They were cut into pieces of about 5 mm × 40 mm.

3. Pharmacological procedure

A piece of the muscle was suspended in a 30 ml organ bath and the responses were recorded on an ink writing kymograph through a lever of ca. 0.5 g. The cumulative technique was mainly used. A set of responses was recorded every 15 minutes and the muscles were washed three times within an interval with Tyrode solution. The bath was filled with Tyrode solution (pH 7.7–7.9) kept at 32°C, which was bubbled with air. The muscle suspended was about 1.0 g in wet weight.

4. Labeling procedure

Labeling of the tissues was performed according to the procedures which were arranged following on the pharmacological experiments. The longitudinal smooth muscle of the cat ileum was cut into small pieces (1–3 cm long), and they were mixed well in Tyrode solution which was bubbled with air. In the case of the other tissues Tyrode solution was gassed with a mixture of 95% O₂ and 5% CO₂. The ratio of wet weight of immersed tissue to the volume of the bath fluid added was kept almost constant throughout the experiments. Labeled organs were minced into ca. 2 mm square, homogenized at ice cold temperature by Waring Blender at a maximum speed for 2 minutes four times after adding 10 volumes of 0.32 M sucrose solution (pH 7.8 with tris(hydroxymethyl)aminomethane) and lyophilized.

5. Measurement of radioactivity

In the case of ³H-Dib a labeled sample was weighed and the tritium was converted into tritiated water by a combustion method established by Tsurufuji et al. (12). Otherwise the sample was solubilized as follows (13). One ml of Hyamine 10-X was added to the sample, moistened again with 0.1 ml water in the counting vial and the vial was kept at 50°C for 1 to 5 hours till the sample was solubilized. After confirming that the solution
in the vial was not coloured brown, the scintillator as mentioned below was added. The counting sample was kept in the sample chamber for more than 12 hours prior to the counting which is enough to abolish the fluorescence due to the solubilized tissue components. When the sample was not solubilized without colouring, the combustion method was applied. In any case the latter method was confirmed to give the same result as the former on the same kind of sample. C\textsuperscript{14}-Dib labeled sample was dispersed in the scintillator with Cab-O-Sil 15 ml, instead of utilizing the combustion method. Before counting the vial was shaked vigorously. One 1 scintillator was composed of 7.0 g PPO, 0.3 g dimethyl POPOP, 100 g naphthalene and dioxane. The radioactivity was counted by Packard Tri Carb liquid scintillation counter, model 3203, maintained at +6°C. A minimum of 10,000 counts was collected for each labeled sample and appropriate backgrounds were determined for each non-labeled tissue sample which was treated in the same way as the labeled sample. The counting efficiency was determined by an internal standardization method and in the case when efficiency was less than 10\%, an external standardization method was applied. The radioactivity was expressed with absolute disintegration per minutes. At least two different amounts of one sample were treated independently and counted. The counting error was less than 2.5\%.

RESULTS

1. Pharmacological responses

A) The response of longitudinal muscle strips compared with intact small intestinal strips

The preparation of longitudinal smooth muscle responded so well to ACh, histamine (Hist), norepinephrine (NE), 5-hydroxytryptamine (5HT) and Dib as the preparation of the longitudinal muscle with the circular muscle or an intestinal strip from which the mucosa was not removed. Responses to ACh were constant at least for 20 hours and those to Hist for 12 hours.

B) Optimal condition of dibenamine incubation in order to block the action of histamine (Fig. 1)

Ten minutes incubation of the muscle with dibenamine 4\times10^-6 \text{M}, and 20 minutes incubation with Dib. 1\times10^-6 \text{M} did not antagonize the action of Hist irreversively. Twenty minutes incubation with Dib. 4\times10^-8 \text{M} and 1\times10^-3 \text{M} antagonized the action of Hist irreversively and this blockade was continued for more than 11 hours. (Fig. 3-A) The contraction induced by 5 HT and the relaxation induced by NE were not so noticeable and 30\% of the muscle preparations tested did not respond to these two agonists. The relaxation by NE was tested in the presence of ACh 10^-7 \text{M}. The responses to NE and 5HT were also antagonized with this condition of Dib incubation. By two repeated 20 minutes incubations of the muscle with Dib. 4\times10^-4 \text{M} the action of ACh was not inhibited but by 20 minutes incubation with Dib 1\times10^-4 \text{M} the action of ACh was antagonized to some extent.

Consequently it was found that responses of the preparation to Hist, 5 HT and NE were blocked but that to ACh was not blocked by 20 minutes incubation with Dib 4\times10^-6 \text{M}. 
Fig. 1. Inhibitory effect of dibenamine on the actions of various agonists.

Dose-response curves were obtained on longitudinal muscle strips of cat small intestines after the incubation with dibenamine. The effect of dibenamine was tested after washing the strip sufficiently till the action of the agonists became constant.

Abscissa: molar concentration of agonist in the organ bath.
Ordinate: contraction height (%) relative to the maximal contraction by acetylcholine.

The horizontal bar indicates S.E. of concentrations of the agonist which gave the 50% contraction height, and the vertical bar S.E. of contraction heights (%).

A: Cumulative dose-response curves for histamine
  a) histamine alone on non-treated muscle strips.
  b) after 10 minutes incubation with dibenamine $4 \times 10^{-6}$M (41 strips from 6 cats).
  c) after 20 minutes incubation with dibenamine $1 \times 10^{-5}$M (36 strips from 5 cats).
  d) after 20 minutes incubation with dibenamine $4 \times 10^{-6}$M (109 strips from 16 cats).
  e) after 20 minutes incubation with dibenamine $1 \times 10^{-5}$M (38 strips from 6 cats).

B: Cumulative dose-response curves for acetylcholine
  a) acetylcholine alone on non-treated muscle strips.
  b) after twice repeated 20 minutes incubations with dibenamine $4 \times 10^{-6}$M (26 strips from 6 cats).
  c) after 20 minutes incubation with dibenamine $1 \times 10^{-5}$M (31 strips from 7 cats).

C: Dose-response curves for norepinephrine
Relaxation by norepinephrine was measured with the inhibition of the contraction produced by acetylcholine $1 \times 10^{-5}$M.
  a) norepinephrine alone on non-treated muscle strips.
  b) after 20 minutes incubation with dibenamine $4 \times 10^{-6}$M (34 strips from 6 cats).

D: Cumulative dose-response curves for 5-hydroxytryptamine
  a) 5-hydroxytryptamine alone on non-treated muscle strips.
  b) after 20 minutes incubation with dibenamine $4 \times 10^{-6}$M (38 strips from 7 cats).
Fig. 2. Protective effect of diphenhydramine, chlorpheniramine and histamine against the action of dibenamine.

Dose-response curves tested on longitudinal muscle strips of the cat small intestines after 20 minutes incubation with dibenamine $4 \times 10^{-4}\text{M}$ in the presence of diphenhydramine, chlorpheniramine or histamine. Protective effects were measured after washing the strips sufficiently till the action of histamine became constant.

Abscissa: molar concentration of histamine in the organ bath.
Ordinate: contraction height (%) relative to the maximal contraction produced by acetylcholine.

The horizontal bar indicates S.E. of concentrations of histamine which gave 50% contraction height, and the vertical bar indicates S.E. of maximal contraction heights (%).

A: Protective effect of diphenhydramine
- a) histamine alone on non-treated muscle strips.
- b) after incubation with dibenamine in the presence of diphenhydramine $1 \times 10^{-6}\text{M}$ (98 strips from 15 cats) or $2 \times 10^{-6}\text{M}$ (29 strips from 8 cats).
- c) after incubation with dibenamine in the presence of diphenhydramine $5 \times 10^{-7}\text{M}$ (35 strips from 5 cats).
- d) after incubation with dibenamine in the presence of diphenhydramine $1 \times 10^{-7}\text{M}$ (28 strips from 4 cats).
- e) after incubation with dibenamine alone.

B: Protective effect of chlorpheniramine and histamine
- a) histamine alone on non-treated muscle strips.
- b) after incubation with dibenamine in the presence of chlorpheniramine $1 \times 10^{-6}\text{M}$ (27 strips from 6 cats).
- c) after incubation with dibenamine in the presence of histamine $8 \times 10^{-5}\text{M}$ (28 strips from 5 cats).
C) Protective effect of diphenhydramine (Diph)

As shown in Fig. 2, four concentrations of Diph were used for protection experiment. Diph was applied 5 minutes prior to the administration of Dib. After sufficient washing, the contraction to Hist became constant as shown in Fig. 3. Diph $1 \times 10^{-7}$ M and $5 \times 10^{-7}$ M did not protect the action of Hist sufficiently from the blocking effect of dibenamine $4 \times 10^{-6}$ M. Diph $1 \times 10^{-6}$ M showed 100% protection of action of Hist from the effect of Dib. Diphenhydramine $2 \times 10^{-6}$ M also showed 100% protection but it took more than 5 hours till the contraction by histamine was fully restored (Fig. 3-C). Under this condition the actions of 5 HT and NE were blocked sufficiently as in the case of incubation with Dib $4 \times 10^{-6}$ M alone. The contraction by ACh was not affected. The incubating condition in which the actions of 5 HT and NE were blocked but the actions of ACh and Hist were not blocked, was 20 minutes incubation of the muscle with Dib $4 \times 10^{-6}$ M in the presence of Diph $1 \times 10^{-6}$ M.

D) Protective effect of histamine (Fig. 2-B)

Hist $8 \times 10^{-7}$ M was applied prior to the addition of Dib $4 \times 10^{-6}$ M and incubated for 20 minutes. After washing the muscle for 1.5 hours, the contraction by Hist became constant. The protection of the action of Hist from the effect of Dib was sufficient but not 100%.

E) Protective effect of chlorpheniramine (CP) (Fig. 2-B)

CP $1 \times 10^{-6}$ M was used for the protection in the same conditions as Diph. After
FIG. 4. Competitive and protective effect of aniline, benzylamine and phenethylamine.

Cumulative dose-response curves for histamine tested on longitudinal muscle strips of the cat small intestine in the presence of the amines and after 20 minutes incubation with dibenamine $4 \times 10^{-6}$m in the presence of the amines. Protective effect was measured after washing the muscle sufficiently till the action of histamine became constant.

Abscissa: molar concentration of histamine in the organ bath.

Ordinate: contraction height (%) relative to the maximal contraction by acetylcholine. The horizontal bar indicates S.E. of concentration of histamine which gave 50% contraction height, and the vertical bar indicates S.E. of maximal contraction height.

A: Competitive effects of the amines
   a) histamine alone.
   b) in the presence of aniline $5 \times 10^{-4}$m (11 strips from 3 cats).
   c) in the presence of phenethylamine $5 \times 10^{-5}$m (12 strips from 3 cats).
   d) in the presence of benzylamine $1 \times 10^{-7}$m (13 strips from 3 cats).
   e) in the presence of benzylamine $1 \times 10^{-8}$m (8 strips from 4 cats).

B: Protective effect of the amines
   a) histamine alone on non-treated muscle strips.
   b) after incubation with dibenamine in the presence of benzylamine $1 \times 10^{-7}$m (12 strips from 3 cats).
   c) after incubation with dibenamine in the presence of aniline $5 \times 10^{-4}$m (11 strips from 3 cats).
   d) after incubation with dibenamine in the presence of phenethylamine $5 \times 10^{-5}$m (10 strips from 3 cats).
   e) after incubation with dibenamine alone.
wearing for 3 hours, nearly 100% recovery of Hist contraction was observed.

F) Effects of aniline (AN) benzylamine (BA) and phenethylamine (PA) on the contraction by histamine (Fig. 4-A)

The three aromatic amines were used for protection, to confirm the correlation between the pharmacological action and incorporated radioactivity. AN $5 \times 10^{-4}$ M antagonized neither the action of ACh nor Hist. PA $5 \times 10^{-4}$ M slightly antagonized Hist and ACh. The actions of NE and 5 HT were not affected. BA $1 \times 10^{-7}$ M, on the other hand, fully antagonized the actions of ACh and Hist.

G) Protective effects of the amines (Fig. 4-B)

Concentrations of the amines mentioned above were used for protection experiments. AN and PA did not protect the action of Hist from blocking effect of Dib $4 \times 10^{-6}$ M. However, BA protected the action of Hist sufficiently but not completely, because the contraction was not restored to a normal level after washing the muscle for more than 5 hours.

H) Action of Dib on the once protected and washed muscle strip

In the muscle which showed full histamine sensitivity after the protection, 20 minutes incubation with Dib $4 \times 10^{-6}$ M blocked completely the action of Hist as shown in Fig. 5, but did not block the action of ACh at all. From these results a procedure for labeling histamine receptor with labeled dibenamine was decided as follows.

At first, non-labeled dibenamine was applied to the control sample without any protective drug and to the test one in the presence of one of protective drugs. After washing the muscle for 3 hours labeled dibenamine was applied to both samples for 20 minutes, washed for 2 hours and then the two preparations were counted for their radioactivity.

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**Fig. 5.** Effect of dibenamine on the longitudinal smooth muscle strips of the cat small intestines which was treated with dibenamine in the presence of diphenhydramine.

Dose-response curves for histamine tested after the second 20 minutes incubation with dibenamine $4 \times 10^{-6}$ M (b) on the muscle strips which showed full histamine sensitivity after the first treatment with dibenamine in the presence of a protective drug (a).

Abscissa: molar concentration of histamine in the organ bath.

Ordinate: contraction height (%) relative to the maximal contraction height by acetylcholine.

The horizontal bar indicates S.E. of maximal contraction heights (%), and the vertical bar indicates S.E. of concentration of histamine which gave 50% contraction height.
Flow Sheet 1. Procedure for specific labeling of histamine receptors in the cat small intestinal muscle (Procedure 1).

Muscle strips

| Test sample | Control sample |
|-------------|----------------|
| non-labeled dibenamine $4 \times 10^{-6}$ M for 20 min in the presence of the drug for protection as shown in Table 1 | non-labeled dibenamine $4 \times 10^{-6}$ M alone for 20 min |
| washing for 3 hr | washing for 3 hr |
| labeled dibenamine $4 \times 10^{-6}$ M for 20 min | labeled dibenamine $4 \times 10^{-6}$ M for 20 min |
| washing for 1 hr | washing for 1 hr |
| non-labeled dibenamine $5 \times 10^{-6}$ M for 20 min, 10 times* | non-labeled dibenamine $5 \times 10^{-6}$ M for 20 min, 10 times* |
| homogenizing at ice cold temperature | homogenizing at ice cold temperature |

* As the carrier of non-reacted dibenamine which might be adsorbed in tissue.

Flow Sheet 2. Procedure for specific labeling of histamine receptors in the cat small intestinal muscle (Procedure 2).

Muscle strips

| Test sample | Control sample |
|-------------|----------------|
| labeled dibenamine $4 \times 10^{-6}$ M alone for 20 min | labeled dibenamine $4 \times 10^{-6}$ M for 20 min in the presence of the drug for protection as shown in Table 2 |
| washing for 3 hr | washing for 3 hr |
| non-labeled dibenamine $5 \times 10^{-6}$ M for 20 min, 10 times* | non-labeled dibenamine $5 \times 10^{-6}$ M for 20 min, 10 times* |
| homogenizing at ice cold temperature | homogenizing at ice cold temperature |

* As the carrier of non-reacted but adsorbed labeled dibenamine

In the test preparation histamine receptor might be labeled with dibenamine. In the control labeled dibenamine reacted only with the other sites than histamine receptor and the difference of radioactivity between the test and control preparation must be the radioactivity of histamine receptor reacted with labeled dibenamine (Flow sheet 1).

The 2nd procedure for specific labeling is as follows (Flow sheet 2). In the control sample labeled dibenamine is applied in the presence of a protective drug and in the test preparation labeled dibenamine alone is applied. With this procedure histamine receptor must be labeled in the test preparation.

2) Radioactivity found in the tissues

A) Diphenhydramine protection in longitudinal muscle of the small intestine

As shown in Tables 1 and 2, significant differences of the radioactivities were observed
### Table 1. Radioactivity found in the longitudinal muscle homogenate according to Procedure 1.

| Drug used for protection | Concentration | Labeled Dibenamine | N* | n** | Test sample | Control sample | Difference*** | % protection**** |
|-------------------------|---------------|-------------------|----|-----|-------------|----------------|--------------|-----------------|
| Diphenhydramine         | $2 \times 10^{-4}$ M | ³H               | 2  | 8   | 35.4±0.9   | 24.6±0.8       | 10.8±0.8  | 44              |
| Diphenhydramine         | $1 \times 10^{-4}$ M | ³H               | 25 | 95  | 33.1±0.5   | 24.5±0.3       | 8.6±0.2   | 35              |
| Diphenhydramine         | $1 \times 10^{-4}$ M | ¹⁴C              | 3  | 14  | 5.3±0.1    | 3.9±0.1        | 1.4±0.1  | 35              |
| Diphenhydramine         | $5 \times 10^{-7}$ M | ³H               | 4  | 8   | 29.6±0.8   | 24.8±0.7       | 4.8±0.4  | 19              |
| Diphenhydramine         | $1 \times 10^{-7}$ M | ³H               | 3  | 9   | 26.5±0.9   | 24.7±0.6       | 1.8±0.3  | 7               |
| Histamine               | $8 \times 10^{-7}$ M | ³H               | 3  | 9   | 31.1±0.5   | 24.3±0.3       | 6.8±0.2  | 21              |
| Chlorpheniramine         | $1 \times 10^{-6}$ M | ³H               | 3  | 8   | 31.8±0.3   | 24.2±0.3       | 7.6±0.2  | 31              |

* 'N' indicates the number of experiments.
** 'n' indicates the number of the cats used.
*** 'Difference' indicates the difference of radioactivity between the test sample and the control sample.
**** '% protection' = \( \frac{\text{Difference}}{\text{dpm of the control sample}} \times 100 \).

### Table 2. Radioactivity found in the longitudinal muscle homogenate according to Procedure 2.

| Drug used for protection | Concentration | Labeled Dibenamine | N* | n** | Test sample | Control sample | Difference*** | % protection**** |
|-------------------------|---------------|-------------------|----|-----|-------------|----------------|--------------|-----------------|
| Diphenhydramine         | $2 \times 10^{-4}$ M | ³H               | 2  | 8   | 33.9±0.9   | 22.3±0.9       | 11.6±0.8  | 34              |
| Diphenhydramine         | $1 \times 10^{-4}$ M | ³H               | 12 | 52  | 33.8±0.6   | 25.3±0.4       | 8.5±0.2  | 25              |
| Diphenhydramine         | $1 \times 10^{-4}$ M | ¹⁴C              | 7  | 24  | 5.5±0.1    | 4.1±0.1        | 1.4±0.1  | 25              |
| Diphenhydramine         | $5 \times 10^{-7}$ M | ³H               | 12 | 25  | 33.9±0.7   | 28.3±0.6       | 5.6±0.3  | 17              |
| Diphenhydramine         | $1 \times 10^{-7}$ M | ³H               | 12 | 25  | 32.1±0.6   | 28.3±0.6       | 3.8±0.3  | 3               |
| Histamine               | $8 \times 10^{-7}$ M | ³H               | 7  | 14  | 34.0±0.6   | 27.4±0.4       | 6.6±0.3  | 19              |
| Histamine               | $8 \times 10^{-7}$ M | ¹⁴C              | 5  | 10  | 5.5±0.1    | 4.8±0.1        | 0.7±0.1  | 13              |
| Chlorpheniramine         | $1 \times 10^{-4}$ M | ³H               | 9  | 19  | 34.0±0.6   | 25.6±0.5       | 8.4±0.4  | 25              |
| Chlorpheniramine         | $1 \times 10^{-4}$ M | ¹⁴C              | 3  | 7   | 5.5±0.1    | 4.2±0.1        | 1.3±0.1  | 24              |
| Benzylamine             | $1 \times 10^{-5}$ M | ¹⁴C              | 3  | 15  | 2.1±0.2    | 3.4±0.2        | 1.3±0.1  | 42              |
| Benzylamine             | $1 \times 10^{-7}$ M | ¹⁴C              | 4  | 32  | 3.9±0.1    | 0.4±0.1        | 0.4±0.1  | 29              |
| Aniline                 | $5 \times 10^{-4}$ M | ¹⁴C              | 4  | 32  | 5.1±0.1    | 0.4±0.1        | 0.4±0.1  | 7               |
| Phenethylamine           | $5 \times 10^{-4}$ M | ¹⁴C              | 4  | 32  | 5.1±0.1    | 0.4±0.1        | 0.4±0.1  | 7               |

* 'H' indicates 'H-dibenamine and '¹⁴C' indicates '¹⁴C-dibenamine.

'Difference' = \( \frac{\text{dpm of the test sample} - \text{dpm of the control sample}}{\text{dpm of the control sample}} \times 100 \).

Between the test and control preparations, when $10^{-6}$ M Diph was used, significant differences were also observed, but the differences increased with the increase of the concentrations of Diph. When Diph $10^{-7}$ M and $5 \times 10^{-7}$ M was used for protection, significant differences were observed. B) Histamine protection and chlorpheniramine protection Nearly the same results were obtained as in the case of protection with Diph $1 \times 10^{-6}$ M. C) Benzylamine protection Even in the case of $10^{-7}$ M of benzylamine greater difference of radioactivity was found than that found in the case of protection with Diph $1 \times 10^{-8}$ M, Hist or CP.
D) Protection with aniline and phenythylamine

There was found little difference of radioactivity in these cases but the differences were still significant.

E) Radioactivity found in other tissues than longitudinal muscle of the small intestine

As the tissues sensitive to histamine, tracheal muscles and heart muscles were chosen. As the tissue with low histamine sensitivity, circular muscles of small intestines of the cat were chosen. As the tissues insensitive to histamine, abdominal muscle and diaphragm were chosen. The liver was used as a control, which is not a contracting tissue. These tissues were incubated according to the two procedures using Diph 1 x 10^{-8} M and/or Hist 8 x 10^{-7} M for protection. Results were shown in Tables 3 and 4. In tissues with higher histamine sensitivity, larger amounts of differences of the radioactivity were observed and in the tissues with low or no histamine sensitivity, little differences were found. The difference was not so small in the liver and was even higher than that in the circular muscles which have low histamine sensitivity.

**Table 3.** Radioactivity found in the several tissues labeled with 3H-dibenamine under the protection with diphenhydramine.

| Tissue                                      | Procedure 1 | Procedure 2 |
|---------------------------------------------|-------------|-------------|
|                                             | (6 experiments on 9 cats) | (5 experiments on 7 cats) |
| Longitudinal muscle of small intestine      | Test sample | Control sample | Difference | Test sample | Control sample | Difference |
| 32.8 ± 0.7                                  | 24.7 ± 0.5  | 8.1 ± 0.3   |
| Tracheal muscle                             | 29.6 ± 0.6  | 25.1 ± 0.6  | 4.5 ± 0.4  |
| Heart muscle                                | 31.1 ± 0.9  | 27.3 ± 0.8  | 3.8 ± 0.3  |
| Liver                                       | 25.7 ± 0.7  | 23.3 ± 0.7  | 2.4 ± 0.2  |
| Circular muscle of small intestine          | 26.8 ± 0.4  | 25.1 ± 0.6  | 1.7 ± 0.4  |
| Diaphragm                                   | 23.6 ± 0.6  | 22.2 ± 0.6  | 1.4 ± 0.8  |
| Abdominal muscle                            | 23.7 ± 0.6  | 22.5 ± 0.7  | 1.2 ± 0.4  |

**Table 4.** Radioactivity found in the several tissues labeled with 3H-dibenamine under the protection with histamine.

| Tissue                                      | Procedure 1 | Procedure 2 |
|---------------------------------------------|-------------|-------------|
|                                             | (5 experiments on 8 rats) | (4 experiments on 6 cats) |
| Longitudinal muscle of small intestine      | Test sample | Control sample | Difference | Test sample | Control sample | Difference |
| 31.5 ± 0.6                                  | 24.5 ± 0.4  | 7.0 ± 0.4   |
| Tracheal muscle                             | 28.1 ± 0.6  | 24.9 ± 0.5  | 3.2 ± 0.3  |
| Heart muscle                                | 31.0 ± 0.8  | 28.6 ± 0.9  | 2.4 ± 0.3  |
| Liver                                       | 24.7 ± 0.7  | 23.1 ± 0.7  | 1.6 ± 0.3  |
| Circular muscle of small intestine          | 25.9 ± 0.7  | 24.9 ± 0.6  | 1.0 ± 0.2  |
| Diaphragm                                   | 23.0 ± 0.8  | 22.4 ± 0.7  | 0.6 ± 0.2  |
| Abdominal muscle                            | 22.9 ± 0.7  | 22.5 ± 0.6  | 0.4 ± 0.3  |
F) The correlation between the incorporated radioactivity and the extent of protected receptors

The protection experiments with various concentrations of Diph in Table 2 were used and the extent of protected receptors was calculated from the dose-response curve according to Furchgott (14). As shown in Fig. 6, sufficient correlation was found between the extent of protected receptors and incorporated radioactivity which was obtained with or without Diph. In the protection with Diph $2 \times 10^{-4}$ M smaller incorporation, that is, greater radioactivity difference than that with Diph $1 \times 10^{-5}$ M was observed though pharmacological protection was complete even in the latter case.

DISCUSSION

The longitudinal muscle layer of the cat small intestine which responds well to histamine is consisted of almost uniform muscle cells. It includes Auerbach plexus and has only a small amount of the connective tissue. The two procedures of labeling histamine receptors will be specific from the pharmacological point of view. In control samples, according to the procedure 1, fairly high radioactivity was found notwithstanding the pretreatment with non-labeled dibenamine. This defect might be diminished when higher concentration of dibenamine was used in the protection experiment with corresponding higher concentration of diphenhydramine. But higher concentration of diphenhydramine could not be completely washed out for more than 5 hours and after that the preparation could not respond normally to histamine and so this procedure was not used for further radiochemical treatment. The smaller radioactivity difference relative to the control sample suggested the presence of a small amount of histamine receptors compared with the total nucleophillic groups reactive to dibenamine. The fact that the radioactivity bound with the control sample of procedure 1 equaled almost that of procedure 2 indicates that it is bound to an unsaturable dibenamine binding site. After the protection and washing of the preparation in procedure 1 the remaining dibenamine binding sites consist of histamine receptors and non-specific nucleophillic groups and the latter will now be in the same conditions for labeled dibenamine as those of procedure 2. Paton suggested the presence of unsaturable atropine binding sites in the
small intestine of the guinea pig (15). It was supposed by Triggle et al. (7, 10) that
cleophilic groups were newly uncovered in smooth muscle tissues after the binding
of dibenamine with already present nucleophilic sites. Either of the ideas is possible
for the elucidation of this phenomenon.

The difference of radioactivity between the test and control which was obtained in
procedure 1 was then compared with that obtained in procedure 2 and it was found that
the two differences were nearly the same. This similarity proves the adequacy and
reproducibility of these procedures.

We tried to protect the histamine receptor with various drugs other than diphenhy-
dramine. Histamine and chlorpheniramine were expected to protect specifically histamine
receptor and really gave the significant differences of radioactivity which were nearly the
same as that in the diphenhydramine protection. Benzylamine which antagonized phar-
macologically against histamine and acetylcholine gave the largest radioactivity difference
among the protective drugs tested. In the case of protection with high dose of aniline or
phenethylamine little but statistically significant differences were observed. These
radioactivity differences should not be specific for histamine receptor but must be ascribed
to radioactivity non-specifically bound with the other sites than histamine receptor.

In these experiments benzylamine protection presented a problem. Benzylamine
1 \times 10^{-7} \text{m} \text{c} could not afford full protection of the action of histamine from the inhibitory
effect of dibenamine, but the radioactivity difference was much larger in this case than in
the case of protection with diphenhydramine, chlorpheniramine or histamine which showed
nearly complete pharmacological protection. With benzylamine 1 \times 10^{-6} \text{m} the radio-
activity difference was larger than the radioactivity found in the control sample, and so
it is supposed that a part of benzylamine protected sites may be histamine receptors and
the other part nonspecific benzylamine binding sites.

Both dibenamine binding sites such as histamine receptor and nonspecific dibenamine
binding sites may be differentiated each other, when distributions of radioactivity differences
in cell fractions of the homogenates protected with various specific or non-specific drugs
are compared (16). Between the extent of pharmacological protection and the incorpo-
rated radioactivity sufficient correlation was obtained (Fig. 6). At 1 \times 10^{-4} \text{m Diph} the
receptor was protected completely and it is a sufficient concentration for the protection.
At 2 \times 10^{-6} \text{m Diph} the radioactivity incorporation was further decreased but it might be
derived from other sites than the histamine receptor. By the procedures mentioned above
some part of non-receptor incorporation of the radioactive dibenamine could be excluded.

Among the tissues of the cat the radioactivity differences obtained similarly in the
treacheal muscle, the heart muscle and the liver were smaller than that of the longitudinal
muscle and larger than those of the circular muscle of small intestine and the skeletal muscle.
The results in Tables 3 and 4 indicate that the incorporated radioactivity differences are
proportional to the histamine sensitivity of the tissues.

All the facts cited above indicate that the radioactivity differences obtained by specific
protection are parallel to amounts of histamine receptors present in the organs and further
a fairly larger part of the difference is occupied by histamine receptors. Of course we does not neglect the existence of the radioactivity difference originated from non-receptor binding, which we are trying to separate from the specifically labeled receptor fraction.

SUMMARY

Histamine receptor of the cat small intestinal smooth muscle was investigated by means of specific alkylation with labeled dibenamine under the conditions determined pharmacologically. In combination with dibenamine several pharmacologically protective and non-protective drugs against dibenamine were used to test the specificity of radiochemical protection of the receptor from the action of labeled dibenamine. The significance of the radioactivity difference between the tissue treated with labeled dibenamine alone and that treated with labeled dibenamine in combination with one of the drugs was discussed. Among the drugs used, diphenhydramine, histamine, chlorpheniramine and benzylamine showed sufficient radiochemical protection and aniline and phenethylamine also showed radiochemical protection eventhough it was weak.

The amounts of the active receptors still remaining after the treatment with labeled dibenamine under various doses of diphenhydramine showed sufficient correlation with incorporated radioactivity.

Several tissues with various histamine sensitivities and without it were treated with dibenamine in combination with diphenhydramine or histamine. The correlation between the histamine sensitivity and the radioactivity difference was confirmed.

These results suggest that the radioactivity difference was parallel to the amount of histamine receptors apparently blocked with labeled dibenamine although non-specific combination might be fairly contaminated in the difference. It is concluded that histamine receptor of the cat small intestinal muscle is labeled rather specifically when this labeling method is applied.

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