PURIFICATION OF HISTAMINE RECEPTOR (VII)
REASSESSMENT OF SPECIFICITY OF RADIOACTIVE
DIBENAMINE TO LABEL H_1 RECEPTORS

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In a foregoing paper (1), a method for labeling histamine H_1 receptor with radioactive dibenamine in the smooth muscle of cat small intestine was proposed. We found 7 p moles bound dibenamine per mg protein of the total tissue homogenate and 40 p moles per mg protein of the plasma membrane fraction. This communication deals with reconsideration on the receptor specificity of this bound dibenamine or, more accurately, on the selectivity of H_1 receptor. The procedure in the previous paper (1) involved 'double protection' which means the two-step application of protectors against dibenamine: the first protection was the usual one with protective antihistamine, promethazine, and the second was cross protection of non-specific sites with drugs having negligible antihistaminic actions in the doses used, thioridazine and atropine. Muscle strips protected with promethazine against non-radioactive dibenamine were treated with 3H -dibenamine in the presence of these second protectors. The second protectors were to cover non-specific sites which had been protected non-specifically with promethazine from non-radioactive dibenamine. The first non-radioactive dibenamine was 4 times higher in concentration than the second 3H -dibenamine.

The difficulty is in determining the proportion of non-specific sites labeled with 3H -dibenamine presumed to represent receptor-specific binding, as reported previously (2). This problem arises from the non-saturable mode of binding of dibenamine to the tissue. Moran et al. (3) reported that new nucleophilic groups were successively uncovered to react with dibenamine after dibenamine had bound to those groups already present. In our method, 3H -dibenamine is considered to bind to the newly uncovered groups even after non-radioactive dibenamine reacts with the non-receptor sites already present. To assess non-receptor binding of 3H -dibenamine, 3H -dibenamine (1×10^{-6} M, whose concentration was sufficient to suppress histamine-induced contraction in our preparations) was applied in the presence of promethazine (3×10^{-7} M), thioridazine (3×10^{-7} M) and atropine (3×10^{-7} M) after application of non-radioactive dibenamine (4×10^{-6} M) under protection with promethazine (3×10^{-7} M) as shown in Fig. 1-A. The radioactivity of bound 3H -dibenamine was compared to the sample from the same cat labeled in the presence of thioridazine and atropine but without promethazine as shown in Fig. 1-B. Thioridazine and atropine did not protect the histamine H_1 receptor under these conditions, however protection was evident with promethazine. The labeled muscle strips were homogenized and fractionated as described previously (4). 3H -Dibenamine (58.5 Ci/mole) was prepared as already reported

FIG 1. Procedures for labeling with ³H-dibenamine. An alley cat was anesthetized with pentobarbital sodium (35 mg/kg) and exanguinated. Small intestines were removed and the longitudinal layer of smooth muscle was carefully peeled off. *Procedure A* was for labeling non-receptor sites which were uncovered after unlabeled dibenamine had bound to non-receptor sites already present. *Procedure B* was for labeling both receptor sites and non-receptor sites. Thus the difference between the two procedures will represent the receptor label whose proportion to non-receptor ones is now under investigation.

(1) and the bound radioactivity was counted after oxidizing the samples in a Model 306 Tri-carb oxidizer (Packard Instrument) (1). We could determine the radioactivity of 0.20 p moles of ³H-dibenamine per mg protein with confidence limits of 0.16–0.22 p moles ($\alpha=0.01$, $N=10$) (5) when the non-labeled muscle homogenate (10 mg protein) on a filter paper was oxidized after addition of 2.0 p moles of ³H-dibenamine. Protein was determined by the micro-biuret reaction (6).

The results are shown in Table 1. Although the values varied widely among the cats, the difference of the amount of bound dibenamine between the samples labeled in the presence of promethazine (*Procedure A*) and those in its absence (*Procedure B*) was 0.9 ± 0.2 p moles per protein of the total homogenate or 3.4 ± 0.8 p moles per mg protein of the plasma

TABLE 1. Bound ^3H -dibenamine to the total homogenate and to the plasma membrane fraction from smooth muscle of cat small intestine

Total homogenate	Bound ^3H -dibenamine (p moles/mg protein)							mean $\pm$ SE.
	1	2	3	4	5	6	7	
Procedure A	2.5	3.6	4.3	5.3	5.9	5.9	6.8	4.9 $\pm$ 0.6
Procedure B	3.6	4.2	4.7	5.6	6.9	7.5	8.2	5.8 $\pm$ 0.6
Difference	1.1	0.6	0.4	0.3	1.0	1.6	1.4	0.9 $\pm$ 0.2
Plasma membrane fraction	I	II	III	IV	V	VI	mean $\pm$ S.E.	
Procedure A	9.2	12.4	19.8	20.2	22.5	23.2	17.9 $\pm$ 2.3	
Procedure B	10.4	16.3	25.8	22.9	23.9	28.6	21.3 $\pm$ 2.7	
Difference	1.2	3.9	6.0	2.7	1.4	5.4	3.4 $\pm$ 0.8	

The muscles were labeled as described in Fig. 1. *Procedures A* and *B* were indicated in the figure and 'difference' means the difference of the amount of bound ^3H -dibenamine between the samples treated according to *Procedure A* and those to *Procedure B*. Standard error (S.E.) for 'difference' was calculated from each value of 'difference'. On the total homogenates, 7 cats were used and on the plasma membranes 6 cats were used. The result from each cat is shown in the column under the numeral (Arabic for the homogenate and Roman letters for the membrane fraction).

membrane fraction. There may have been a bulk of dibenamine bound to non-receptor sites in our previous trial (1) because of the non-saturable mode of dibenamine binding.

Hill *et al.* (7) used ^3H -mepyramine for binding studies in the whole homogenate of the smooth muscle of guinea pig small intestine and reported that the specific binding was 0.05–0.3 p moles per mg of the homogenate (8). We studied the binding of ^3H -histamine to the plasma membrane fraction of the smooth muscle of cat small intestine, in which the binding capacity was 1.0–2.5 p moles per mg protein of the fraction. The K_D value was $1\text{--}3 \times 10^{-7}$ M (2). These values were obtained by applying a model with two orders of sites to the Scatchard plots and neglecting the sites with the highest affinity. But when these high affinity sites were picked out and plotted reciprocally according to Lineweaver-Burk equation, the binding capacity was 0.2–1.0 p moles per mg protein of the membrane fraction and K_D was $0.3\text{--}1.0 \times 10^{-8}$ M. Thus our previous values might have been overestimated. Although these binding data were obtained *in vitro* on dispersed cells or on their sub-fraction, these values were about 1/2–1/15 of the present results obtained by application of the irreversible antagonist to the living tissue. Thus, even with precautions there are limitations to using radioactive dibenamine. However, such do provide a criterion for further fractionation of labeled materials or for another trial to label H_1 receptor with a more selective alkylator. For example, diphenhydramine mustard (9) has about a 7-fold higher affinity for H_1 receptor than dibenamine whose apparent affinity for H_1 receptor after 20 min incubation is about 1/40 of mepyramine.

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