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An Investigation Into The Mechanism Of Action Of H(1)-Histamine Receptor Antagonists On Histamine Release From Rat Peritoneal Cells.

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AN INVESTIGATION INTO THE MECHANISM OF ACTION OF H_1-HISTAMINE RECEPTOR ANTAGONISTS ON HISTAMINE RELEASE FROM RAT PERITONEAL CELLS

by

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A DISSERTATION

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ATP.....adenosine triphosphate

c-AMP.....cyclic 3'5' adenosine monophosphate

c-GMP.....cyclic guanosine monophosphate

DFP.....diisopropylphosphofluoridate

DSCG.....disodium chromoglycate

ECF-A.....eosinophil chemotactic factor of anaphylaxis

EC_{50}.....concentration of a drug which inhibits a response by 50%.

ED_{50}.....dose of a drug which inhibits a response by 50%

EDTA.....ethylene diamine tetraacetate

F_{ab}.....fraction of papain-digested antibody molecule containing a light chain and a variable portion of a heavy chain

F_c.....constant portion of the fraction of papain-digest antibody molecule containing part of the heavy chains

HCl.....hydrochloric acid

HEPES.....N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid

IgA.....immunoglobulin A

IgD.....immunoglobulin D

IgE.....immunoglobulin E

IgG.....immunoglobulin G

IgM.....immunoglobulin M

M.W.....molecular weight

OPT.....o-phthaldialdehyde

PAF.....platelet activating factor

PCA.....passive cutaneous anaphylaxis

PNU.....protein nitrogen units

RPMI.....Roswell Park Memorial Institute

S.E.M.....standard error of the mean

SRS-A.....slow reacting substance of anaphylaxis

2-DG.....2-deoxy-D-glucose

$\bar{X}$.....mean

INTRODUCTION

Even at the present time, the mechanism of the allergic response and its characterization remain obscure (61,79,96). From the time von Pirquet defined allergy (293), tremendous advances have been made, especially in the study of extrinsic hypersensitivity (245). The demonstration that homologous antibodies could passively sensitize cells (178,230) and produce a hypersensitive response (219) led to the characterization of histamine release in the mast cell (139) and basophil (172). The current level of knowledge allows an intelligent use of these systems to evaluate selected ways of modifying the allergic condition (7,45,50,137,170,174). The present experiments define in more detail the mechanism of histamine release and how it is inhibited by several drugs.

Historical

About 1920, Manwaring and co-workers (179-189,216) demonstrated that the liver was the anaphylactic shock organ in the dog (292,297-299). Histamine (1,153,235) was identified and found to be present during the anaphylactic response (64,65). It was, also, about this time that Coca and Grove (46) characterized many of the features of the reaginic antibody which causes the anaphylactic response and is currently classified as IgE (Table 1).

Table 1

Characteristics of IgE and Skin Fixing (Reaginic) Antibody

	IgE*	Reaginic Antibody
Molecular weight (M.W.)	190,000	—
Sedimentation rate	8.2	8
Carbohydrate (%)	11.5	—
Light chain (M.W.)	λ(22,600)	k,λ
Heavy chain (M.W.)	ε(72,500)	—
Antibody activity	+	+
Sensitized homologous tissue	+	+
Heat (56°C, 30 min)	labile	labile
Reduction of sulfhydryl bonds	labile	labile
Complement binding	0	0
Electrophoretic mobility	γ1	γ1

* IgE = IgND, a myeloma protein

This table is from Bennich and Johansson (24)

Characterization and Structure of Immunoglobulin E (IgE)

Although the enormous diversity in antibody specificity was known (72, 100,256,278), the structural similarites of the monomers of different classes of antibodies (70,71,90,97,104,106) (Table 2) was not appreciated until about 1960 when the antibody molecule was first dissociated into its 4-chains (68). In the 1960's, the production of an immunoglobulin was selectively induced (204,205) which had the characteristics of reaginic antibody (110,114,115). This new class of antibodies was named IgE (113) since it differed from the other classes of antibodies (IgG, IgD, IgA, IgM) (108,109,110). Although one subclass of IgG antibodies can cause histamine release (10,11), IgE is the only immunoglobulin that selectively binds to mast cells (93,122,151,154,157,165) and basophils (19,116,169,194,289,290) of the same species (13,107,220), but not heterologous species (121,203,227,233,302), for a long period of time to produce an allergic response (155,197,206,210,221) on antigen challenge (52,246,298). Since IgE has a short serum half-life (287, 294) and is found at low serum concentrations in normal individuals (23,132), it was only after an IgE myeloma protein was discovered (24) that its structure could be studied (152) and related to cell binding (62,259). Activation of the cell by antigen bridging of two IgE molecules (12,69,149,240) produced mediator release (51,162,265) and a somewhat slower congregation of the cell-bound immunoglobulin molecules at one pole of the cell (156,263). An extensively hydrophobic

Table 2

Classes of Human Immunoglobulins*

Class	γG	γA	γM	γD	γE
Heavy chains:					
class	γ	α	μ	δ	ε
subclasses	γ1, γ2, γ3, γ4	α1, α2	——	——	——
M.W.	53,000	64,000	70,000	——	75,000
Light chains:					
M. W. 22,500	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
S_{20}, w	6.5-7.0	7,10,13,15,17	18-20	6.2;6.8	7.9
M.W.	150,000	180,000-500,000	950,000		196,000
Carbohydrate %	2.9	7.5	11.8		10.7
Concentration in normal human serum, mg/100 ml	800-1,680	140-420	50-190	3-40	0.01-0.14

* This table was modified from Edelman and Gall (72). The nomenclature used is that recommended by the World Health Organization.

receptor for IgE (107,119) made up of two or more sub-units (48,83) has been isolated from the basophil membrane (109,117,123,212).

Mast Cells and Basophils

Ehrlich, better known for his work in chemotherapy (60,61) is cited for the initial identification of the mast cell (193,248) and basophil (59,244). However, there is still disagreement on exactly how to differentiate them from other cell types (222,223,255). The metachromatically staining granules of these cells contain three mediators of the allergic responses: heparin (66,126,127,237,305), histamine (47,91,190,213,238,239) and serotonin (21,22,28,241,247, 249,263,279). Three other agents which mediate a response associated with anaphylaxis -- eosinophil chemotactic factor of anaphylaxis (ECF-A) (44,94,143,144,264,280,281,295,296), slow reacting substance of anaphylaxis (SRS-A) (14,33,34,37,38,147,217,267) and platelet activating factor (PAF) (25,26,27,32,142,158,166,258,259) may or may not be released by mast cells and basophils.

Mediator Release from Mast Cells and Basophils

The present discussion of mediator release deals almost exclusively with histamine since its release has been studied in much more detail than that of the other allergic mediators.

Extracellular Factors Affecting Histamine Release

The characteristics of allergic histamine release appear to be the same for any manner of stimulating the IgE receptor whether it be the allergic antigen/antibody reaction (111,112,119,120,164,266), anti-IgE against either the F_{ab} or F_c portion or the whole IgE molecule (211,276). In sensitized cells, the amount of histamine released appears to depend primarily on the intracellular enzymatic and metabolic status of the cell rather than the degree of sensitization (49,118,232,303). Little is known about the reaction between the cell surface and the start of the events leading to histamine release inside the cell (162), but certain extracellular conditions are necessary for histamine release. In general, histamine release from the mast cell and basophil occur under similar conditions, although the rate of release is different (150,172).

Incubation Temperature and Histamine Release

Optimal release from both the mast cell and basophil occurs at 37°C (13,169,199); the rate decreases at other temperatures and is abolished above 45°C or below 20°C (31,195,198). Although heating does not appear to affect cell-bound antibody, it can antagonize further binding of IgE to cells (150,198).

Temperature is also an important factor in histamine release by the

non-cytotoxic chemical histamine releasing agents, and the histamine release by these agents also occurs at a maximum rate at 37°C (134,145). While the morphologic and histamine releasing pattern is similar for Compound 48/80 and antigen-induced histamine release (30), there are differences in the mechanism of stimulation since Compound 48/80 can desensitize mast cells to IgE-mediated histamine release but the converse does not occur (54,260).

Extracellular Ionic Requirement for Histamine Release

Divalent cations are required for histamine release from mast cells and basophils. The most important ion appears to be calcium (172); however, it can be mimicked to some extent by strontium (85) or an increase in pH (200). Besides calcium, an extracellular source of energy is also required for histamine release by the antigen/antibody reaction, Compound 48/80, or the calcium ionophore A23187 (55,138, 209,224). The optimal calcium level for histamine release in the antigen/antibody reaction is 1 to 2 mM (76,87). The chemical releasing agents require a slightly lower concentration of calcium (80,224); however, strontium does not replace calcium as effectively with the chemical releasing agents as in antigen-induced release, resulting in a slower release even at high concentrations of strontium (80,82,87).

Intracellular Steps in Histamine Release

A chronological sequence of events for the intracellular steps for the release process has been presented for the mast cell (Fig. 1) and for the basophil (Fig. 2). Although the steps in these sequences do not follow the same order for the two cell types, they are reported to have the same components and, since the current study is of the mast cell, the sequence presented for the mast cell will be followed.

Serine Esterase Activation

Calcium influx appears to activate a serine esterase in both the basophil (173) and the mast cell following the reaction of antigen with cell-bound antibody (58,140). The activation of this enzyme by the calcium influx induced by either the antigen/antibody reaction, Compound 48/80, or the calcium ionophore A23187 can be completely antagonized by the serine esterase inhibitors, di-isopropylphosphofluoridate or by phenylmethylsulphonylfluoride (58).

Energy Requirement for Histamine Release

The next step in this sequence was an energy requiring step which can be antagonized by 2-deoxyglucose (Fig. 1). Glucose (87,41) or tissue glycogen (214,215) which is converted to ATP (adenosine triphosphate) (55,191) is the energy source for histamine release by the antigen/antibody reaction (130,131), Compound 48/80 (202,229) or A23187 (128, 129). Extracellular energy is more important during the slower re-

Fig. 1 Sequence of events in mast cell histamine release. The sequence of events recognized in the histamine release process are listed with the blocking agents used to help identify these steps. Diisopropyl-phosphofluoridate (DFP), 2-deoxy-D-glucose (2-DG). ethylene diamine tetraacetate (EDTA), cyclic adenosine monophosphate (c-AMP), cyclic guanosine monophosphate (c-GMP). Solid lines are either direct chemical reaction, or movement of a substance or ion across a membrane. Dashed lines indicate other steps in the process which require, or are modulated by the cofactors mentioned.

The diagram is modified from Kaliner and Austen (139).

Figure 1

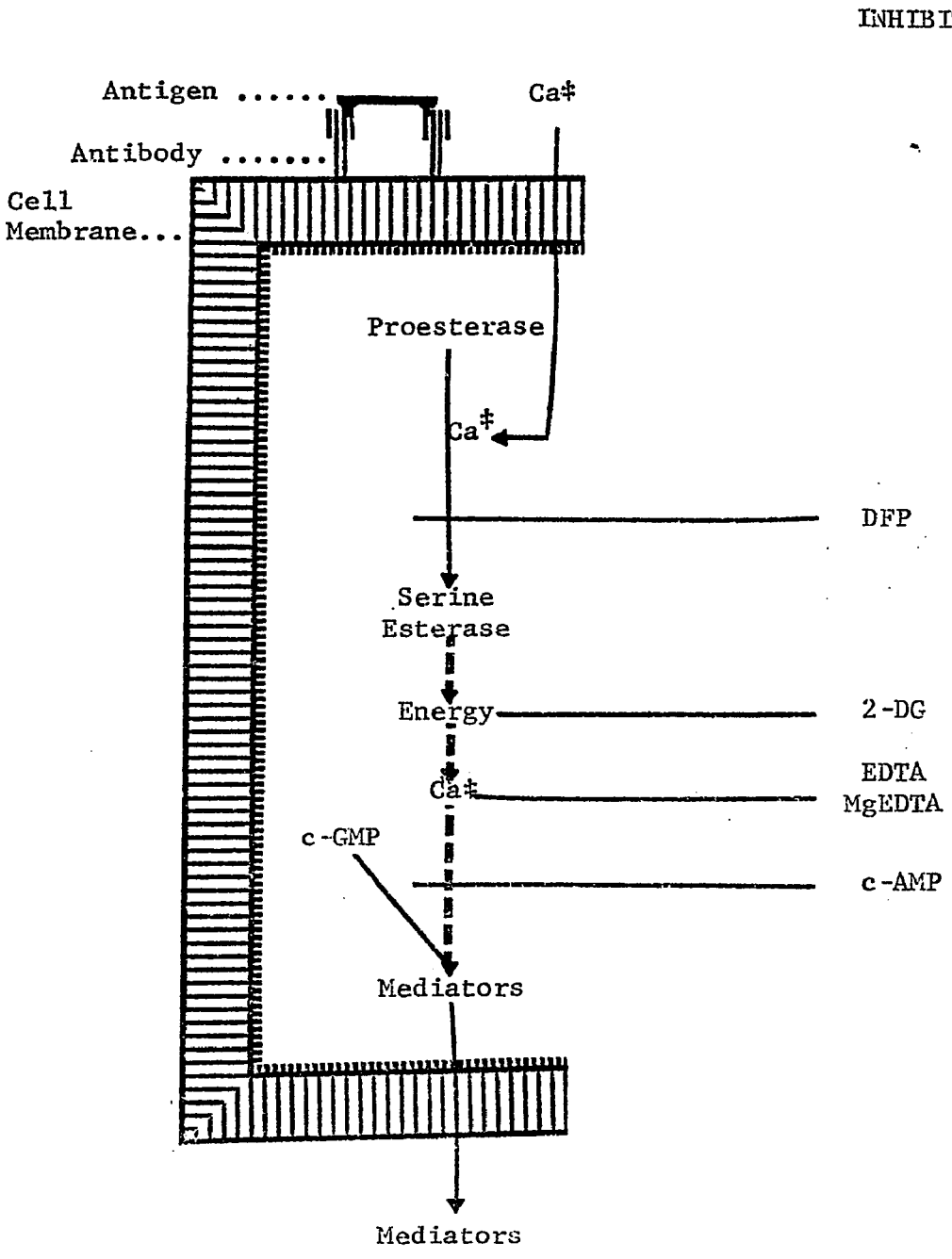
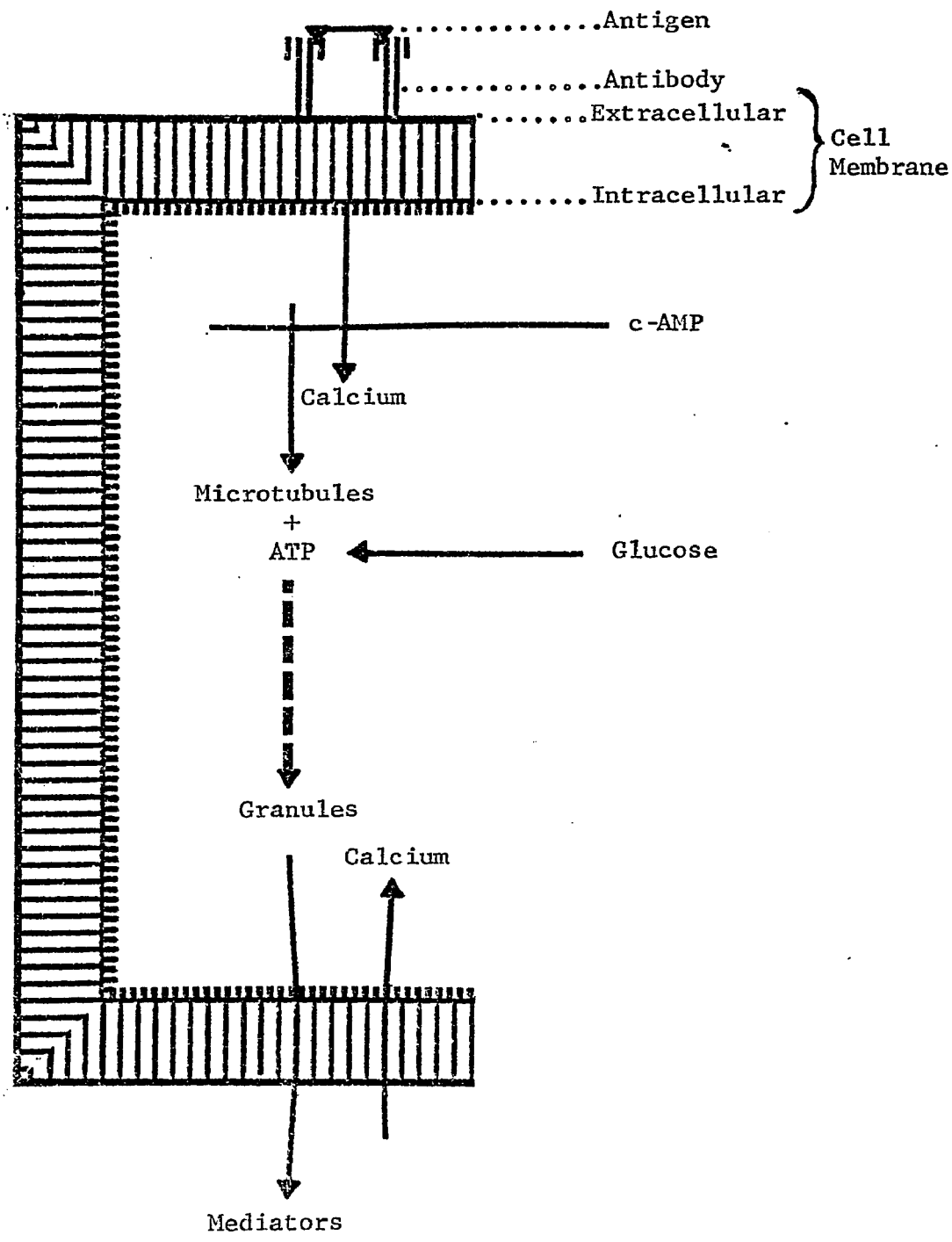

INHIBITORS
Antigen .....
Ca‡
Antibody .....
Cell
Membrane...
Proesterase
Ca‡
DFP
Serine
Esterase
Energy
2-DG
EDTA
MgEDTA
Ca‡
c-GMP
c-AMP
Mediators
Mediators

Fig. 2 Sequence of events in histamine release from the basophil.

This diagram was modified from Lichtenstein (168).

Figure 2



BASOPHILS

lease process from the basophil (219) since a continuous energy source is required during this process.

Effect of Cyclic Nucleotides on Histamine Release

A step affected by cyclic nucleotides comes immediately after the energy requiring step (Fig. 1) and is strongly affected by it. c-AMP (cyclic 3′5′ adenosine monophosphate) does not antagonize histamine release from well-nourished cells as effectively as from those where the energy supply has been depleted (89,214,215). The effect of c-AMP is also closely related to the second calcium-dependent step (the first being the influx initiating the intracellular sequence of events resulting in histamine release), and has not actually been separated from it in either the mast cell (140,272), or basophil (35,92). No effect of c-AMP was observed on unsensitized cells when antigen was administered (102) or on the membrane permeability of unchallenged sensitized cells (270); therefore, this step comes later than the first calcium-dependent step (Fig. 1) (271). c-AMP levels remain elevated for a more prolonged period of time in calcium-free than calcium-containing medium (214,272) and dibutyryl c-AMP inhibits histamine release and calcium uptake to about the same extent (83,102,270) indicating that this cyclic nucleotide may affect intracellular calcium mobilization (92).

When mast cells are stimulated by the antigen/antibody reaction,

Compound 48/80 or calcium ionophore A23187, there is a rapid increase in c-AMP which begins to decrease prior to histamine release (175,180). An increased c-AMP level will antagonize histamine release in the basophil (8,35) or mast cell following challenge with antigen (261) or Compound 48/80 (271). The general consensus is that high concentrations of c-AMP inhibits histamine release (214,215); however, this may simplify this interaction too much. Gillespie and Lichtenstein indicate that the effect of c-AMP in modulating histamine release may not be related specifically to either a high or low concentration, but to a correct concentration (92).

Histamine Release from Mast Cell Granules

The granule has been recognized as the most likely source of histamine in the mast cell (77) and basophil (238,239,253) since it was definitely shown that these cells contained histamine. Histamine can be released from mast cell granules by relatively mild procedures which do not contain sufficient energy to break covalent chemical bonds (283). Although some histamine release has been reported to occur through channels (161) without granule exocytosis (261,262), usually release occurs from the charged granules (4,303) after exocytosis (20,43,208). The histamine is replaced by cations (56,277,288) in the ion exchange matrix of the granule (285,286,287). The total cell is required for histamine release by antigen/antibody reaction, or Compound 48/80; however, A23187 can release histamine from the isolated granule (99).

Modification of Histamine Release with Drugs

Several types of drugs and many chemical compounds can either induce or inhibit histamine release. Some of the sites that can be affected by various agents are shown in Figure 1. When the extracellular membrane is perturbed, histamine release occurs. These agents can affect the cell membrane by either a destructive effect, such as that produced by detergents (134) or a modification with non-cytotoxic agents which cause a calcium influx (101,159).

Antagonism of histamine release from the cell can be accomplished by agents which antagonize mast cell or basophil activation, or inhibit any of the subsequent steps in the release process. Each of the intracellular steps which are recognized can be inhibited by specific chemicals (Fig. 1), however, these steps are found in many body systems and therefore their inhibition would be of a non-specific nature. Agents which inhibit the release process specifically in cells which contain the mediators of the allergic response would be potentially useful in controlling allergic diseases.

Agents Affecting c-AMP and Histamine Release

There are specific receptors on the mast cell or basophil which can either initiate or modify histamine release. A step affected by the cyclic nucleotides can be modulated by agents such as the catecholamines (171,268) or prostaglandins (175) which stimulate adenylate

cyclase to increase c-AMP formation, or the xanthines which inhibit the destruction of cyclic nucleotides by antagonizing phosphodiesterase activity (102).

Agents Affecting Calcium Influx and Histamine Release

A receptor on the cell surface is known to initiate allergic histamine release (212); however, little is known about the process at the membrane (88) except that it is related to a calcium influx.

The method of introducing calcium into the cell does not appear to be a critical factor since calcium injected into the mast cell with a microelectrode causes histamine release. The specificity for calcium, however, was shown in that no histamine was released when either potassium or magnesium ions were injected into the mast cells using the same microelectrode techniques (141).

Agents which have an effect on the calcium influx in mast cells or basophils will affect histamine release. Some agents antagonize the movement of calcium across membranes of cells which contain allergic mediators, as well as other cell types such as muscle (75,86). Therefore, these compounds will antagonize histamine release but are not specific enough to be therapeutically useful.

Disodium Chromoglycate (DSCG): Use in Asthma

Disodium chromoglycate is the most recent addition to the therapeutic arsenal for the treatment of asthma (105,201,226). This compound does not affect IgE levels in body fluid (282), nervous control of respiration (124,125) or the response to mediators of the allergic reaction (251). One problem with this compound is presenting a sufficient amount to the site of action, therefore, DSCG is administered by insufflation (225) which may cause bronchospasm (133).

DSCG Antagonism of Histamine Release

DSCG will antagonize mediator release from sensitized mast cell preparations (40,50,95) and mast cells challenged with non-toxic concentrations of Compound 48/80 (3,57,218). The inhibition of histamine release by DSCG is accompanied by a concomitant antagonism of radioactive calcium uptake in mast cells demonstrating an inhibition of release at the calcium entry stage of release (84). This compound is only effective in the mast cell and does not antagonize histamine release from basophils (7); therefore, all discussion of the antagonism of histamine release by DSCG in this report is related to work in the mast cell.

H_1-Histamine Receptor Antagonists in Allergy

The receptors for histamine in the body have been divided into two categories, H_1 and H_2, based on the ability of different agents to duplicate or antagonize selected responses to histamine (29) (Table 3).

Antihistamines (36,53) antagonize the effects of histamine on some tissues (63,242); however, when the concentration of these agents is sufficient, they can also stimulate histamine release (5,262,306). Although there are some reports of beneficial effects by high concentrations of cyproheptadine in allergic-type conditions (192,196, 250), antihistamines are not used in many allergic conditions because of the concentration of agent required to produce an effect. Also, H_1-antihistamines are much more effective against added histamine than against antigen in antagonizing the effect on the bronchial contraction (63). The second reason that antihistamines are not used is probably something of a misconception as stated in one of the most popular pharmacology textbooks, Goodman and Gilman, that "The antihistamines do not prevent histamine-liberating drugs from releasing histamine; indeed, some antihistamines themselves possess histamine liberating properties." (63). Since the first demonstration that drugs could release histamines (2), the experiments of various investigators have confirmed the second portion of this statement (5,170,207); however, the first portion has been disproven in both the basophil (170) and the mast cell (175,304).

Table 3 *

Differentiation of Histamine Receptors

	H_1	H_2
Physiologic agonist	Histamine	Histamine
Specific synthetic agonist	2-Pyridineethanamine	4-Methylhistamine
Antagonist	Chlorpheniramine Pyrilamine Cyclizine Cyproheptadine Diphenhydramine Promethazine	Metiamide
Physiologic action of agonists	Pro-Inflammatory 1) smooth muscle contraction 2) vasodilation 3) increase capillary permeability	Anti-Inflammatory 1) decrease histamine release 2) decrease lymphocyte killing 3) decrease lysosomal enzymes
Mechanism	Increase c-GMP	Increase c-AMP

*This table is modified from Durant et al. (67) and Lichtenstein (168)

H_1-Antihistamines and Histamine Release

In appropriate concentrations, several antihistamines will either inhibit the re-uptake of histamine by mast cells (6) or will protect the mast cells from challenges and keep them from releasing histamine (207,300). The only published studies currently available on this effect of H_1-antagonist in mast cells is one by Mota and DaSilva (207) and a brief communication by Zeppa and Hemingway (306). Mota and DaSilva used only pyrilamine and diphenhydramine as representative agents of the categories of antihistamines used in the present experiments. In their studies, these two agents were effective at concentrations of 0.1 to about 0.5 mg/ml in antagonizing histamine release from guinea pig lung; however, this system releases histamine by an IgG-type of antibody reaction rather than IgE (207). This is at variance with the effect of DSCG which is not an effective antagonist of IgG-mediated histamine release from guinea pig lung (40,50). Mota and DaSilva (207) also reported that the histamine release was not antagonized except at fairly high concentrations which approximated the concentration range where these agents would induce histamine release. They found that the release of histamine caused by antigen or 48/80 in rat and guinea pig tissue was antagonized by both diphenydramine and pyrilamine; however, they did not find that this occurred at less than 0.25 - 1.0 mg/ml (207). Since the rat system used by these investigators probably contained IgE antibodies, this study is the only one available that is closely re-

lated to the objectives of the present experiment.

H_2-Histamine Receptors and Histamine Release

Exogenous histamine will antagonize antigen-induced histamine release from the basophil. This is blocked by H_2- (174), but not by H_1-antihistamines. Actually, the antagonism of antigen-induced histamine release by a combination of exogenous histamine and the H_1-antihistamines is greater than the effect of either compound alone (170). Antagonism of antigen-induced histamine release by exogenous histamine from human leukocytes is accompanied by an increase in c-AMP (174). However, exogenous histamine does not cause an increase in c-AMP levels in the mast cell (98). In contrast, the H_1-antihistamines caused a slight but significant decrease in the c-AMP level of human leukocytes (170). Exogenous histamine may increase antigen-induced histamine release (42,103) or suppress release (174) in different systems. However, both effects are mediated by the H_2-histamine receptor. Metiamide is reported to not have any effect on antigen-induced histamine release from rat lung (42).

Studies on the Mechanism of Action of H_1-Antihistamines as Antagonists of Histamine Release

Although there is a large body of literature on the extracellular physiochemical conditions and some of the intracellular biochemical processes in histamine release, little has been reported concerning

the triggering mechanism at the cell membrane. Histamine release can be initiated in several ways; however, a specific mechanism of antagonizing release from mast cells at the membrane has been demonstrated for DSCG (50,240,243). The present work demonstrated that: 1) the antihistamines also antagonize histamine release at the cell membrane; 2) that this inhibition is different from that of DSCG; 3) the antagonism of histamine release by the antihistamines was not through either the classical H_1- or H_2-receptor; 4) and, no relationship could be found between a local anesthetic type of membrane stabilization and antagonism of histamine release.

The use of selective agonists of histamine release with the antagonists studied led to the hypothesis that the perturbation of the membrane to allow an influx of calcium is a multi-step process with a possible sequence of events discussed.

MATERIALS AND METHODS

Chemicals

Chemicals, of reagent grade, used were as follows: calcium chloride, sodium chloride, sodium hydroxide, aluminum hydroxide, methanol, 1-butanol (J.T. Baker Chemical Co., Phillipsburg, NJ); hydrochloric acid (Eastman Kodak, Rochester, NY). Heptane was obtained from Eastman Kodak and o-phthaldialdehyde (OPT) from Schwartz Mann, Orangeburg, NJ; Trypan Blue dye (Allied Chemical Co., Morristown, NJ); Evans Blue dye (Matheson Coleman Bell, Norwood, OH); hydrochloride and phosphate salts of histamine† (Nutritional Biochemicals, Cleveland, OH); heparin sodium (Schwartz Mann) were also purchased. The following were donated by the companies supplying them: calcium ionophore A23187† (Eli Lilly and Co., Indianapolis, IN) and Compound 48/80† (Burroughs Wellcome Co., Research Triangle Park, NC). The antigen used throughout these experiments was the soluble extract of pig ascaris purchased from Greer Laboratories, Lenoir, NC.

The following agents were the generous gifts of their respective sources: cyclizine hydrochloride† [M.W. 306.85] (Burroughs Wellcome Co., Research Triangle Park, NC); cyproheptadine hydrochloride† [M.W. 350.89] (Merck Sharp & Dohme, West Point, PA); D-chlorphenira-

† Structures of these compounds are presented in Appendix A

mine maleate† [M.W. 390.86] (Schering Corp., Bloomfield, NJ); diphenhydramine hydrochloride † [M.W. 291.81] (Parke, Davis & Co., Detroit, MI); disodium chromoglycate† [M.W. 584.41] (Fison Ltd., Leichestershire, England); 4-methylhistamine dihydrochloride† [M.W. 198.09] and 2-pyridineethanamine dihydrochloride † [195.09] (Smith Kline & French Laboratories, Philadelphia, PA); metiamide† [M.W. 244.37] (Merrell-National Laboratories, Cincinnati,OH); promethazine hydrochloride † [M.W. 320.87] (Wyeth Laboratories, Philadelphia, PA); and, pyrilamine maleate † [M.W. 401.47] (Sandoz A.G., Basel, Switzerland).

Solutions and Buffers

A Vanlab (San Francisco, CA) pH 7.0 buffer and Leucatrol (Pfizer Diagnostics, New York, NY) were used for daily instrument calibrations. Isoton II® and Zap Isoton II® (Coulter Diagnostics Inc., Hialeah, FL) were diluents used for counting nucleated cells. Roswell Park Memorial Institute (RPMI) medium 1640 was purchased from Grand Island Biological Co., Grand Island, NY. The contents of this medium, as obtained from the supplier, are presented in Appendix B.

RPMI 1640 was modified before use by the addition of calcium chloride to a final calcium ion concentration of 1.0 mM. Three molar HCl was added to the RPMI 1640 until a pH of 6.70 was obtained.

† Structures of these compounds are presented in Appendix A

The modified RPMI 1640 was used as the solvent to make fresh drug solutions for addition to cells within 2 hours of the experiment each day, except for the calcium ionophore. The calcium ionophore was prepared in a fresh batch of modified RPMI 1640 each week and resuspended with an ultrasonic probe daily before use. Metiamide solutions were made by adding the drug to modified RPMI which was acidified and kept at 37°C for 2 hours. The solution was titrated to approximate neutrality with sodium hydroxide and brought to the desired drug concentration with warm (37°C) modified RPMI. The challenge solutions were made by addition of this solution to the necessary volume of warm modified RPMI which contained the desired amount of releasing agent.

The aqueous solutions used in the histamine extraction and assay were dissolved in glass distilled water. Histamine standard stock solutions were stored after dissolving histamine dihydrochloride in distilled water at the beginning of the experiment. This solution was kept at -20° until it was used. There was no decrease in fluorescence readings of this sample for the duration of the experiment.

Labware

All labware that came in contact with peritoneal cell suspensions was disposable polypropylene.

All labware that came in contact with organic solvents during histamine extraction and assay was glass, except for the Teflon® linings on the caps of the glass culture tubes. All non-disposable glassware used was machine-washed and rinsed with deionized water.

Antiserum Production

Rats, weighing 225-275 gm, were sensitized with 15,000 protein nitrogen units (PNU) of ascaris antigen (Greer Laboratories) conjugated to 1.0 mg of aluminum hydroxide injected intraperitoneally along with 10^{10} killed Bordetella pertussis organisms. The serum was harvested two weeks later. The blood, collected after the rats were decapitated, was allowed to stand for 30 minutes at room temperature in polystyrene test tubes. After any adhesions between the clot and the tube was broken, the tubes of blood were kept refrigerated for 2 hours at 5°C. The serum was removed after centrifugation for 15 minutes at 1000 x g.

The serum from each rat was kept separate, and tested at a 1:16 dilution in the rat 48-hour PCA (passive cutaneous anaphylaxis) test as described in the following section (204). The sera, giving a wheal of 5 mm or greater diameter, were combined and the pooled sera titered. All sera pools used in these experiments gave a 5 mm or greater diameter wheal at a PCA titer of 1:64. All pooled sera were stored at either -20° or -70°C and used within 6 months of harvesting.

Passive Cutaneous Anaphylaxis (PCA)

Reaginic content of sera collected from actively sensitized rats was assayed by PCA reactions (204). One-tenth ml of serum diluted in saline was injected, in duplicate, intradermally to determine whether reaginic antibodies were present, or as an alternative procedure, the serum antibody titer was determined by making serial dilutions of serum (from 1:16 to 1:256) which were injected intradermally into the shaved dorsal skin of normal rats. Forty-eight hours later, the animals were injected by cardiac puncture with 1 ml of Evans Blue (5 mg/ml) containing ascaris antigen (4000 PNU/ml) in saline. Forty-five minutes after antigen injection, the animals were killed by cervical dislocation, the skin everted, and the orthogonal wheal diameter measured on the serosal surface of the skin. The PCA titer was the reciprocal of the highest dilution giving an average diameter of 5 mm or greater.

Testing Antiserum for IgE Antibodies

The pooled serum was heated at 56°C for 4 hours and then tested in the rat 48-hour PCA to determine whether it would passively sensitize skin mast cells for antigen-induced histamine release (252). Unheated serum from the same pool was used as a positive control in the same experiments.

With unheated serum, antigen challenge induced histamine release from passively sensitized peritoneal cells and a positive PCA response at 48 hours.

Peritoneal Cell Harvesting

The effect of animal size on the parameters studied was evaluated in a preliminary study. The optimal results were obtained with male Sprague-Dawley rats weighing 250-400 gm, as had been reported by other investigators (9,76). Rats were decapitated and the peritoneal cavity was opened by careful midline abdominal incision. Five ml of modified RPMI 1640 were instilled into the peritoneal cavity and, after gentle massage of the area for 1 minute, the fluid was transferred by a polypropylene pipette into a polypropylene centrifuge tube. The number of cells was determined after dilution in Isoton II® using a Coulter Counter® and the red blood cell contamination was determined after treatment of a portion of the cell suspension with Zap Isoton II® which lysed non-nucleated cells.

Cell Differentials and Viability

The viability of the cell population was determined by making a count of 500 nucleated peritoneal cells suspended in a solution of Trypan Blue. Trypan Blue was prepared as a 0.2% stock solution in distilled water and, immediately before use, four parts of this stock solution

werediluted with one part of a concentrated saline (4.25% sodium chloride) to make it isotonic (73). The dye was mixed with the cells immediately prior to the cell count being made and at least 500 cells from each pooled peritoneal cell population were counted to determine the percentage and viability of mast cells. No cell populations were used unless the viability of both the total cell population and that of the specific mast cell population was greater than 95%. The concentration of mast cells ranged from 4.8 to 5.4% of the total cell count.

Mast Cell Serum Treatment

After the cells were obtained from the peritoneal cavity, they were washed twice in the modified RPMI 1640 medium by being suspended in this medium and sedimented at 200 x g at 4°C for 10 minutes in an IEC PR-6000 refrigerated centrifuge (International Equipment Company, Needham Heights, MA). The peritoneal cells were then suspended in either normal rat serum or antiserum prepared as described above which had been undiluted except for the addition of 50 mcg/ml of heparin (stock solution 1.0 mg/ml). Cells challenged with the chemical releasing agents were incubated in normal serum prior to addition of the agonist in order to: 1) maintain conditions similar to the milieu found in vivo; 2) maintain spontaneous release at relatively low levels (3.9 ± 0.2% [$\bar{X}$ ± S.E.M.] observed with sensitized cells); 3) maintain cell viability. Normal serum treatment is reported to have no effect on histamine release by antigen or the chemical

agonists (99,148,173,228).

Mast Cell Sensitization

The method of mast cell sensitization used was similar to that used by other investigators (13,77). Briefly, the cells were sensitized by keeping them suspended, by periodic shaking, in heparinized serum at 37°C for 2 hours. As reported by other investigators (77), serum kept for a prolonged period of time would still sensitize rat skin for the 48-hour PCA; however, the ability of the serum to sensitize mast cells deteriorated. For this reason, serum was kept at -70°C until it was used, or for a maximum of 6 months, then discarded.

Mast Cell Challenge

After the two-hour incubation of the peritoneal cells with reaginic or normal serum, modified RPMI 1640 was added and the cells were pelleted by centrifugation for 10 minutes at 200 x g at 4°C to remove the serum. The mast cells were then resuspended in a sufficient volume of the modified RPMI 1640 to give a final concentration of 4×10^5 mast cells per ml based on the total cell count and the differential. The cell suspensions were then divided into 2 ml aliquots and incubated in polypropylene tubes at 37°C for 10 minutes. A similar warming period was used for any solutions which were to be used to challenge the cells. For every experiment, all samples were run in

duplicate, and in some cases, quadruplicate. Spontaneous histamine release was determined in cell samples challenged with the medium only. The maximum amount of histamine released by the agonist was determined by challenging the cell with the appropriate agonist in the absence of inhibitor. When the effect of an inhibitor was tested, the quantity of the antagonist and releasing agent contained in the 0.5 ml which was added to the 2.0 ml cell suspension gave the desired final concentration of drug and releasing agent in 2.5 ml. Although there is some relationship between the amount of immunoglobulin E present on mast cells and the antigen concentration necessary to cause histamine release (231), it has been found that there is a range of concentrations of antigen which will produce a near maximal histamine release from a single population of mast cells (160). In the present experiments, ascaris antigen was used to challenge sensitized peritoneal cells and optimal antigen concentrations of 300 PNU/ml was found to produce 20-40% histamine release with an average of 26% ± 0.9 ($\bar{X}$ ± S.E.M.).

Differences in purification procedures or handling and cellular responsiveness, as well as drug treatment, can affect histamine release (123,275). Due to the potential of unexplainable variability, some investigators set standards for an acceptable range of cellular responsiveness to determine whether an experiment should be included in a certain data pool (160). In the present experiments, if hista-

mine release was outside the range of 20-45% of the total histamine content of the cells, the experiment was considered invalid. This is an important consideration with the chemical releasing agents since they have been shown to be lethal to cells at high concentrations (74,135).

Releasing agents were tested in preliminary experiments to determine what concentration was necessary to induce histamine release to the same degree as that produced by the antigen/antibody reaction. It was found that both Compound 48/80 and the calcium ionophore A23187 would cause the desired amount of release at a concentration range of approximately 0.5-1 mcg/ml. The histamine release in these experiments caused by Compound 48/80 was 32% ± 2.3% and the histamine release caused by the calcium ionophore A23187 was 34.6% ± 5.7% ($\bar{X}$ ± S.E.M.). Under these conditions of histamine release, the releasing agent did not damage cells as determined by the ability of the cells to exclude Trypan Blue dye.

Histamine Extraction

The extraction and assay of histamine was carried out, basically, according to the method of Shore et al. (254). After separation from the supernatant, the cells were resuspended in a volume of modified RPMI 1640 to the original volume of 2.5 ml. The supernatants and cell suspension were then placed in a boiling water bath for 15 minutes

to extract the residual histamine from the cells (304). When the material from which histamine is extracted does not contain as much protein, then the total extraction procedure before the histamine assay may be eliminated (28). Since the mast cell suspension contains a relatively small amount of protein, the extraction was initiated with a modification of the second, or wash, step of Shore's procedure. Two ml of either the boiled supernatant or cell suspension was added to glass tubes which contained 0.1 ml of 5N sodium hydroxide, 10 ml of n-butanol and approximately 1.5 gm of sodium chloride, an amount in excess of that necessary to saturate the aqueous portion of the solution. The tubes were then stoppered with caps which had Teflon® liners and thoroughly mixed in an Eberbach reciprocal shaker (Ann Arbor, MI) for 2 minutes. The suspension was then separated into the aqueous and organic phases by centrifugation for 10 minutes at 1000 x g. Using glass pipettes, 8 ml of the upper or butanol phase of these tubes was transferred to a new set of tubes containing 15 ml of heptane and 3.5 ml of 0.1N hydrochloric acid. After this was mixed thoroughly in the Eberbach reciprocal shaker for 2 minutes, the phases were separated by centrifugation at 1000 x g and the organic phase was removed by suction.

Fluorometric Operations

Preliminary experiments indicated that, under the conditions used, none of the agents except 4-methylhistamine caused an effect on the fluorescent assay. This agrees with the results of other investigators (170,306).

Histamine Assay

One ml aliquots of the 0.1N hydrochloric acid phase remaining in the extraction tubes were placed in a new set of tubes. A volume of 0.2 ml of 1.0 N sodium hydroxide and 0.1 ml of a 1% OPT was added, and after 5 minutes, the reaction was stopped by adding 0.1 ml of 3.0 N hydrochloric acid. For control purposes, duplicate samples of the media and duplicate samples of histamine dihydrochloride in the media were carried through all extraction and assay steps starting with the boiling phase. Each solution was then transferred to a quartz cuvette and the fluorescence of the sample was read at an emission wave length of 450 mμ after being activated at an excitation wave length of 360 mμ in a spectrophotofluorometer (Amico-Bowman, American Instrument Co., Inc., Silver Springs, MD).

Calculation of Histamine Release

The quantity of histamine was calculated for each sample from the histamine standard run with each experiment after correction for the

appropriate blank. The mean histamine content of the mast cells used in these experiments was found to be 12.8 mcg of histamine per 10^6 mast cells. This compares favorably with the value reported in the literature of 13 mcg/10^6 mast cells (284). The percent of histamine released by a releasing agent (R) was calculated according to the following formula: $\frac{S}{S+C} \times 100 = R$, where "S" equals the histamine in the supernatant corrected for spontaneous release, and "C" equals the histamine remaining in the cellular fraction. When antagonists of histamine release were used, the percent inhibition (I) was calculated by the following formula: $\frac{Ra-Rx}{Ra-Ru} \times 100 = I$, where "Ra" is the percent release in the absence of antagonist, "Rx" is the percent release in the presence of antagonist and "Ru" is the spontaneous release.

Modification of Histamine Aerosol Lethality in Guinea Pigs

An apparatus modified from that described by Lowe et al. (177) was used to challenge guinea pigs with a histamine aerosol. A chamber fabricated from plexiglass which measured 30 x 30 x 16 cm was divided into four equal compartments by a coarse wire mesh. The coarse wire mesh was also used to make a floor under the animals approximately 1.5 cm from the bottom of the chamber. The chamber was completely sealed with the exception of a round hole in the base directly below the quadrant of the four compartments, and the top which was hinged but not sealed so that vapors could escape from the juncture of the top and the sides.

A DeVilbris #40 nebulizer was secured into the floor of the chamber with an airtight seal. This total apparatus was placed in a fume hood specially modified for this type of testing. A pressure valve system incorporated in the hood and connected to the nebulizer by rubber tube could be set to yield a reproducible air pressure through the nebulizer from day to day.

Hartley guinea pigs of either sex (Murphy Breeders Inc., Plainfield, IN) were dosed orally with either saline or drugs dissolved in saline. The oral dosing was directly into the stomach through a curved ball-tip 7½ cm 16-gauge oral feeding needle (Popper and Sons, Inc., New Hyde Park, NY). A geometric progression of drug doses was administered to the animals one hour before challenge. One animal, at each of 3 dose levels of the compound studied, plus one control animal, were placed in the four compartments of the chamber. The animals received a 2% aerosol of histamine diphosphate (equivalent to a 0.55% solution of histamine base) at 0.36 kg per square cm (5 lb per square inch) air pressure, regulated by the valve system through the DeVilbris #40 nebulizer. In preliminary studies, several parameters affected by histamine aerosols on guinea pigs were examined. The effect of the antihistamine as antagonists of lethality caused by the histamine aerosol was chosen for this report, not only because it was the most definitive, but also because 48 out of 48 control animals died under the condition of these experiments within 5 minutes. Air flow through

the fume hood was sufficient to remove rapidly all of the histamine escaping from the chamber and to quickly remove any histamine from the chamber at the end of each 5-minute experimental period.

The Local Anesthetic Effects of Antihistamines

The relative potency of antihistamines as local anesthetics was determined using the technique of Bulbring and Wajda (39) in guinea pigs. Hartley strain, 200-300 gm, guinea pigs of either sex were obtained from CAMM Research, Wayne, NJ. Drugs were tested at geometrically increasing concentrations based on the percentage of the drug as a salt dissolved in saline. The ED_{50} over the linear portion of the response curve was evaluated to determine the drug concentration necessary to produce a local anesthetic effect to 50% of the stimuli.

Statistical Analysis

In all cases where only two points were compared, this was done by a Student's t-test (301). When more than two points were compared for experimental protocols with a series of modifications of one variable, such as drug concentration or time of administration, the comparison was by Newman-Kuel's multiple comparison test (301). A probit analysis was used to determine median effects (i.e. EC_{50}) of quantal data (78).

RESULTS

Inhibition of Histamine Release from Rat Peritoneal Cells by Several H_1-Receptor Antagonists

Since it had been reported by Lichtenstein and Gillespie (170) that promethazine, among the antihistamines, was one of the most potent inhibitors of antigen-induced histamine release from sensitized human basophils, confirmation of this potency in isolated sensitized rat peritoneal cells was necessary as a prelude to mechanism of action studies. Thus, the potency of a representative antihistaminic agent from each chemical category described by Roth and Tabachnick (242) was examined. When each compound was added to the cell suspension simultaneously with antigen, the rank order of potency was found to be promethazine >cyproheptadine > chlorpheniramine > diphenhydramine > pyrilamine > cyclizine. Promethazine and cyproheptadine were significantly more potent than the other agents tested ($p < 0.05$) (Table 4). Therefore, both of these agents and DSCG were chosen for a detailed comparative study on the histamine release process.

Effect of Promethazine and Cyproheptadine on Spontaneous Histamine Release

Of initial import was the desirability of determining the effect of spontaneous histamine release from rat mast cells in addition to their effect on antigen-induced release since the former process is suggested to be calcium independent whereas the major component of

Table 4

Effect of H_1-Receptor Antagonists on *In Vitro* Antigen-Induced Histamine Release from Passively Sensitized Rat Peritoneal Cells

Drug	Antagonism of Histamine Release EC_{50}* (mcg/ml)
Promethazine hydrochloride	4.7
Cyproheptadine hydrochloride	12.1
Chlorpheniramine maleate	26.0
Diphenhydramine hydrochloride	32.7
Pyrilamine maleate	40.3
Cyclizine hydrochloride	57.5

* Concentration inhibiting antigen-induced histamine release by 50% under optimal release conditions as described in the Methods.

Each point was calculated from 4 to 6 experiments

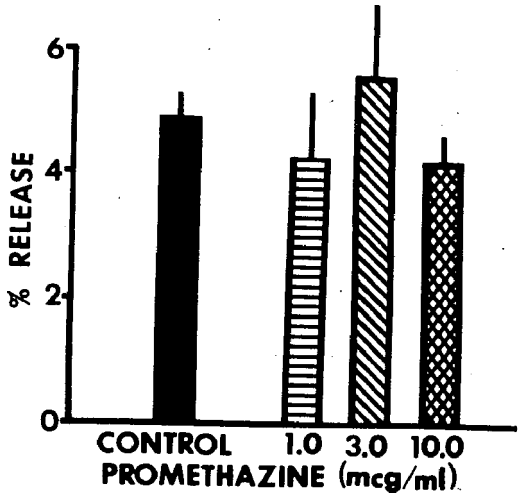
antigen-induced release is calcium dependent (86).

It can be seen in Figure 3 that neither promethazine nor cyproheptadine affected spontaneous release when incubated with peritoneal cells for 5 minutes in concentrations that inhibit antigen-induced release within the same time period ($p > 0.05$). It is worthy to note, at this point, that all previous investigations of DSCG have shown no effect on spontaneous release (304). These results provided the initial clue to some similarity of these two compounds to DSCG and a specificity to the antigen-induced release process.

Duration of Action of Cyproheptadine, Promethazine and DSCG

Kusner et al. (160) have demonstrated that DSCG is not an effective antagonist of antigen-induced histamine release when peritoneal cells are exposed to the drug 10 minutes or more before antigen challenge. Moreover, this was shown not to be due to a decrease in the concentration of DSCG in the medium. In the present study, antigen-induced histamine release was antagonized by about 50% when 3.0 mcg/ml of DSCG was added with antigen. The chromone did not antagonize histamine release when antigen was added 15 to 60 minutes after DSCG (Fig. 4) ($p > 0.05$), confirming the results of Kusner et al.(160). When passively sensitized peritoneal cells were incubated with promethazine or cyproheptadine either 15, 30, or 60 minutes before or

Fig. 3 Effect of promethazine and cyproheptadine on spontaneous histamine release from rat peritoneal cells *in vitro* during a 5 minute incubation period. Each bar represents the mean of four experiments ± SEM.


% RELEASE
6
4
2
0
CONTROL
1.0
3.0
10.0
PROMETHAZINE (mcg/ml)

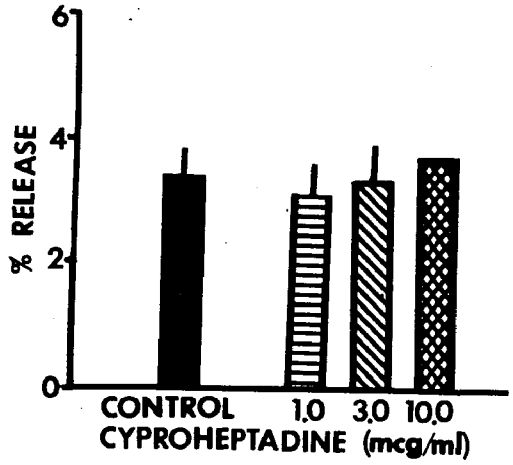
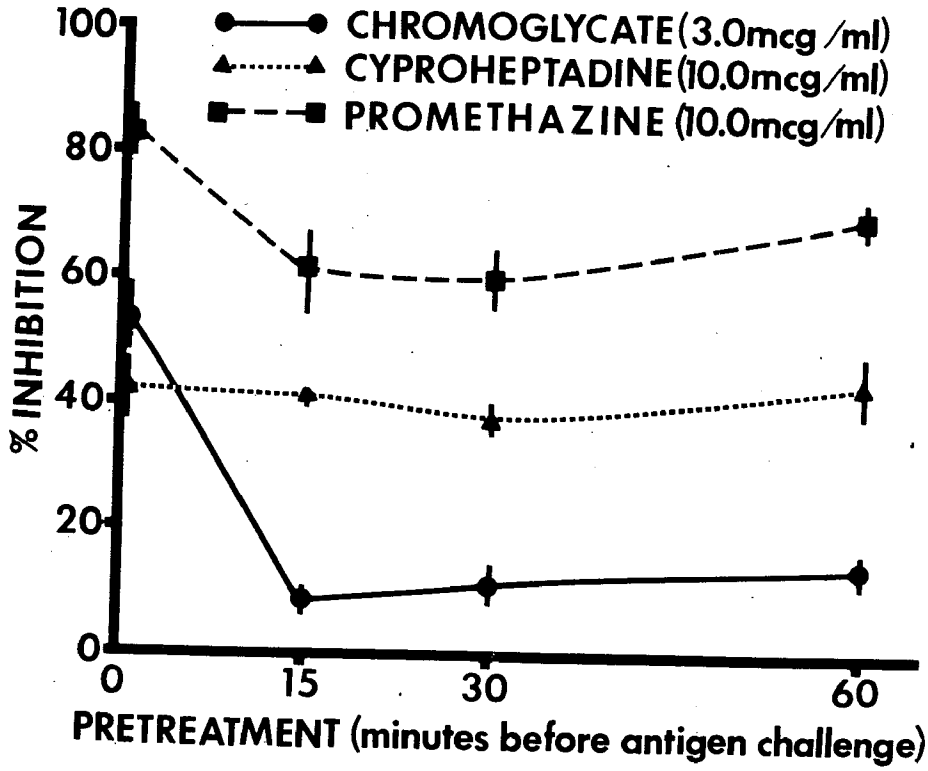

% RELEASE
6
4
2
0
CONTROL
1.0
3.0
10.0
CYPROHEPTADINE (mcg/ml)

Fig. 4 Comparison of the duration of inhibition by DSCG, promethazine and cyproheptadine of antigen-induced histamine release in isolated passively sensitized rat peritoneal cells. Zero time indicates simultaneous addition of drug and antigen. The time periods 15, 30, and 60 minutes indicate the duration of contact of cells with drug before antigen challenge. Each point represents the mean of 6 determinations. Bars indicate SEM.


CHROMOGLYCATE (3.0mcg /ml)
CYPROHEPTADINE (10.0mcg/ml)
PROMETHAZINE (10.0mcg/ml)
% INHIBITION
100
80
60
40
20
0
0
15
30
60
PRETREATMENT (minutes before antigen challenge)

simultaneously with antigen, the inhibition of histamine release persisted (Fig. 4). Cyproheptadine (10 mcg/ml) antagonized antigen-induced release by about 40% when it was administered either with antigen or before antigen. The effect of promethazine (10 mcg/ml) declined moderately with pretreatment time ($p < 0.05$). The effect of DSCG was nearly abolished by pretreatment; however, promethazine retained 75% of its effectiveness, and cyproheptadine retained its full inhibitory effect, under the same conditions.

The Tachyphylactic Effect of DSCG on Antigen-Induced Histamine Release: Comparison with Promethazine and Cyproheptadine

To further evaluate the effect of pretreatment on antigen-induced release, promethazine and cyproheptadine were examined under conditions that produced tachyphylaxis to DSCG. In preliminary experiments, it was found that low concentrations of DSCG, which caused only modest inhibition of histamine release, would not induce a marked tachyphylactic effect. DSCG, at a concentration of 10 mcg/ml, inhibited histamine release by 80% when administered with antigen; however, the antagonism was less than 5% when DSCG was administered 15 minutes before antigen (Fig. 5). Thus, the effects of this concentration were qualitatively similar to those of the lower concentration (Fig. 4). In comparison to a single treatment at the time of antigen challenge, there was essentially no inhibition of histamine release on simultaneous addition of antigen and DSCG to cells which had been previously incubated

Fig. 5 Tachyphylaxis to the DSCG inhibition of antigen-induced histamine release in isolated rat peritoneal cells. Cells were exposed to DSCG in a concentration of 10 mcg/ml 15 min prior to antigen (-15 min), simultaneously with antigen (with Ag), or both. Each bar represents the mean of four experiments ± SEM.

The statistical evaluation is presented in table 5.

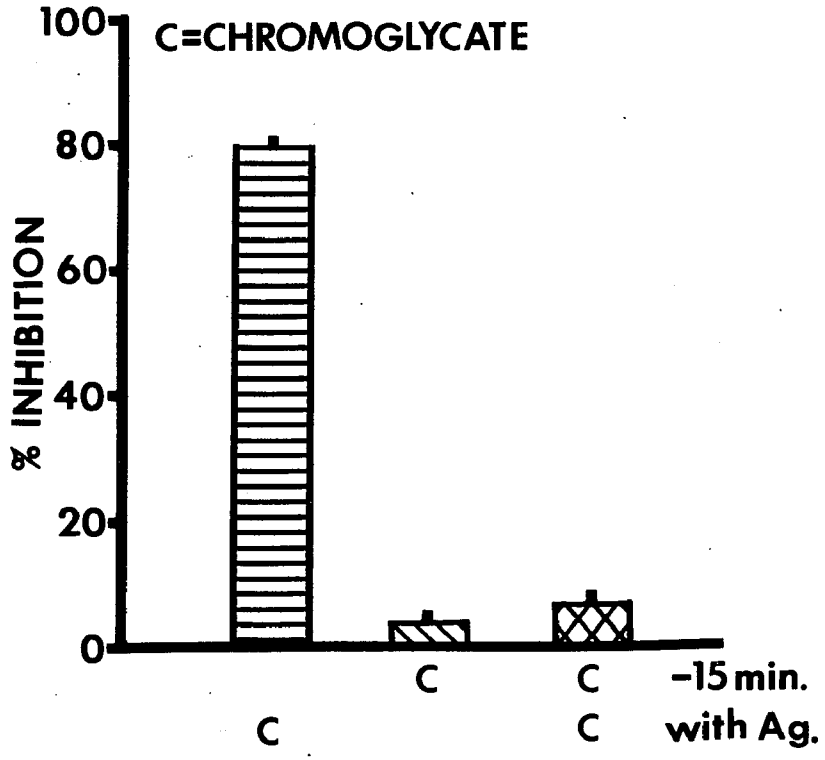

C=CHROMOGLYCATE
100
80
60
40
20
0
% INHIBITION
C
C
C
C
-15 min.
with Ag.

Table 5 A statistical evaluation of interaction between DSCG and cyproheptadine. A single concentration of each compound (10 mcg/ml) was administered either 15 min before or with antigen challenge. An asterisk (*) indicates a significant difference of means between two sets of experimental conditions ($p < 0.05$) as evaluated by the Newman-Kuel's multiple comparison test. Agents administered were disodium chromoglycate (DSCG) and cyproheptadine hydrochloride (cypro). The agents above the line, in each case, were administered 15 minutes before antigen challenge, and agents administered with challenge are recorded below the line. A dashed line (----) indicates when medium alone was added.

	----/DSCG	DSCG/----	DSCG/DSCG	----/Cypro	Cypro/----	Cypro/Cypro	DSCG+Cypro/----	DSCG/Cypro	Cypro/DSCG	----/DSCG+Cypro
MEAN	80.4	4.5	7.2	39.1	38.2	44.6	28.2	35.9	93.7	91.1
----/DSCG		*	*	*	*	*	*	*	*	*
DSCG/----				*	*	*	*	*	*	*
DSCG/DSCG				*	*	*	*	*	*	*
----/Cypro									*	*
Cypro/----									*	*
Cypro/Cypro							*	*	*	*
DSCG+Cypro/----									*	*
DSCG/Cypro									*	*
Cypro/DSCG										
----/DSCG+Cypro										

for 15 minutes with DSCG ($p < 0.05$) confirming tachyphylaxis to DSCG does occur (Fig. 5).

Similar experiments were carried out with cyproheptadine and promethazine at concentrations of 10 mcg/ml. It can be seen in Figure 6 that a second application of cyproheptadine given with antigen challenge did not cause a significant change in the inhibition of histamine release from that already achieved by the first application of drug ($p > 0.05$); thus, no tachyphylaxis with this antihistamine.

In the case of promethazine, the time response studies presented earlier demonstrated that its effect as an antagonist of histamine release decreased about 25% when incubated with cells for 15 minutes prior to antigen challenge. Figure 7 shows that the effect of a double exposure to promethazine, the first 15 minutes before and an additional one with antigen challenge, caused a greater inhibition of histamine release than that produced by a single exposure to the drug at either time period ($p < 0.05$). This demonstrated that promethazine, unlike DSCG, did not exhibit tachyphylaxis. If some minor tachyphylactic effect had occurred, it would be difficult to detect because of the persistent blockade by promethazine when added to cells prior to antigen.

Fig. 6 Comparison of the inhbition of antigen-induced histamine release from isolated rat peritoneal cells by one or two applications of cyproheptadine. Cells were exposed to drug in a concentation of 10 mcg/ml 15 min before antigen (-15 min), simultaneously with antigen (with Ag) or both. Each bar represents the mean effect obtained in four experiments ± SEM.

The statistical evaluation is presented in table 5.

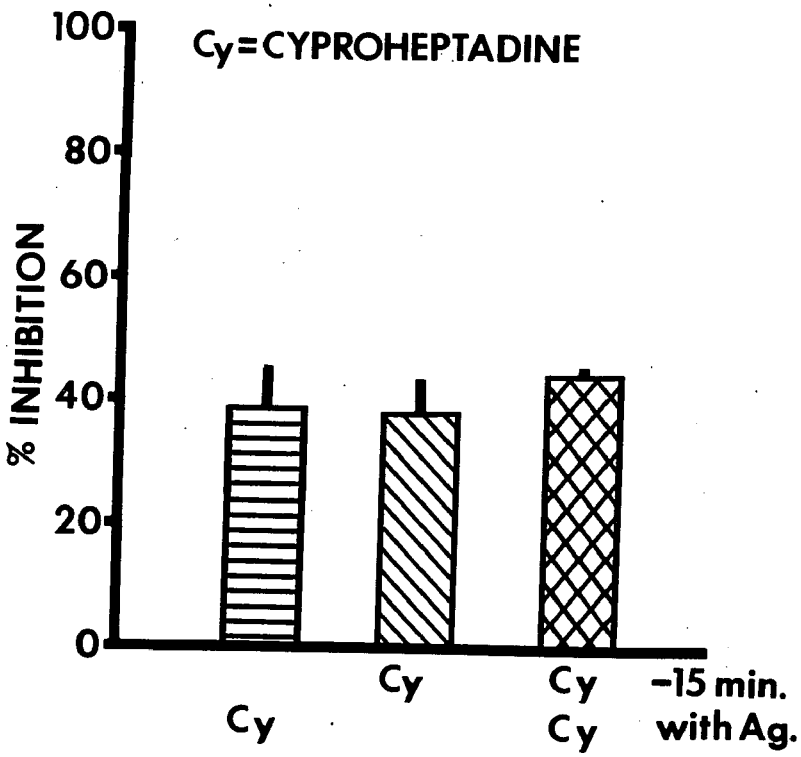

Cy=CYPROHEPTADINE
100
80
60
40
20
0
% INHIBITION
Cy
Cy
Cy
Cy
-15 min.
with Ag.

Fig. 7 Comparison of the inhibition of antigen-induced histamine release from isolated rat peritoneal cells by one or two applications of promethazine. Cells were exposed to drug in a concentration of 10 mcg/ml 15 min before antigen (-15 min), simultaneously with antigen (with Ag) or both. Each bar represents the mean of four experiments ± SEM.

The statistical evaluation is presented in table 6.

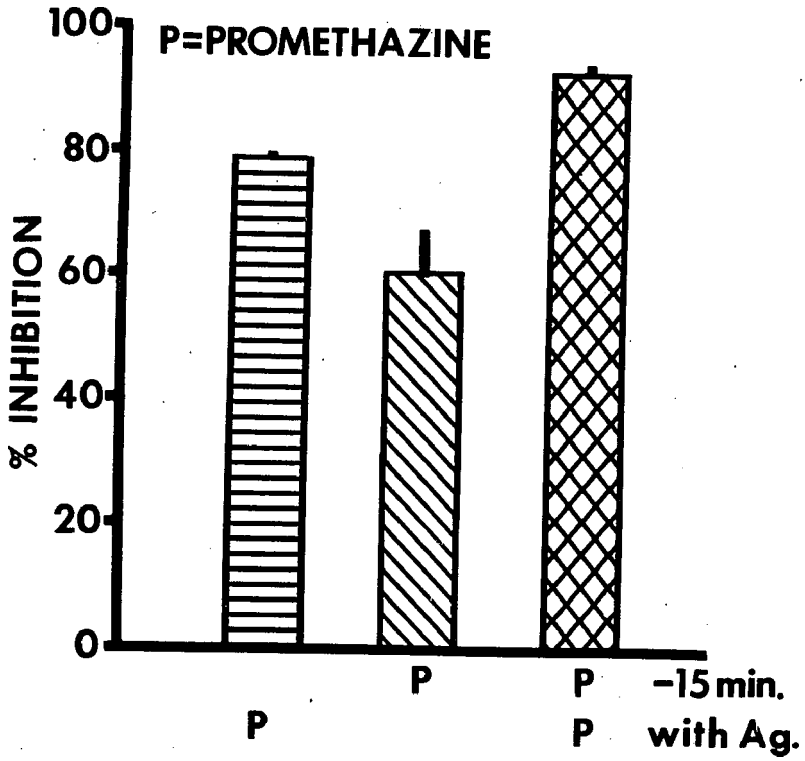

P=PROMETHAZINE
100
80
60
40
20
0
% INHIBITION
P
P
P
P
-15 min.
with Ag.

Table 6 A statistical evaluation of interaction between DSCG and promethazine. A single concentration of each compound was administered before or with antigen challenge. The agents above the line, in each case, were administered 15 minutes before antigen challenge, and under the line, the agents were administered with antigen. Dashed line (----) indicates when medium only was added. DSCG = disodium chromoglycate (10 mcg/ml) and PROM = promethazine hydrochloride (10 mcg/ml). An asterisk (*) indicates a significant difference of means between two sets of experimental conditions ($p < 0.05$) as evaluated by the Newman-Kuel's multiple comparison test.

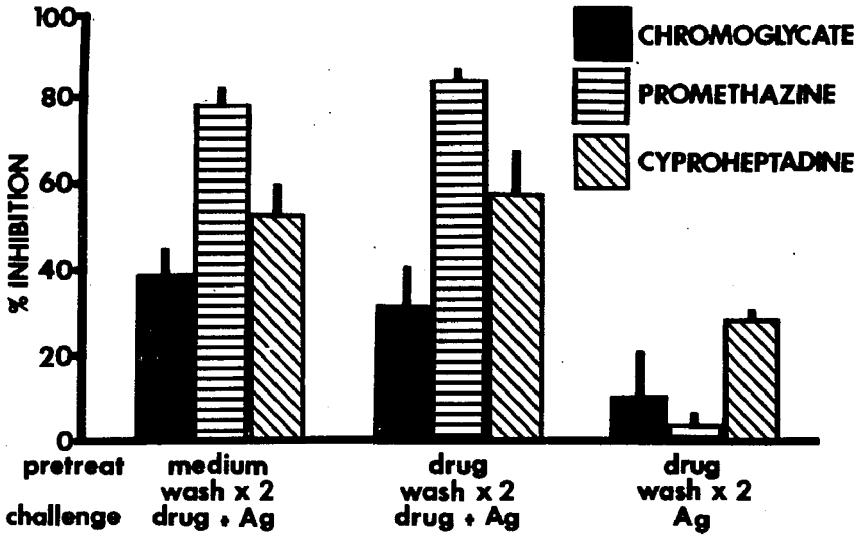
	---- DSCG	DSCG ----	DSCG DSCG	---- PROM	PROM ----	PROM PROM	DSCG+ PROM ----	DSCG PROM	PROM DSCG	---- DSCG+ PROM
MEAN	80.4	4.5	7.2	79.0	60.4	92.9	77.8	81.7	96.3	95.8
---- DSCG		*	*		*	*			*	*
DSCG ----				*	*	*	*	*	*	*
DSCG DSCG				*	*	*	*	*	*	*
---- PROM					*	*			*	*
PROM ----						*		*	*	*
PROM PROM							*	*		
DSCG+ PROM ----									*	*
DSCG PROM									*	*
PROM DSCG										
---- DSCG+ PROM										

Reversibility of Drug Effects by Washing

Sung et al. (273,274) have reported that the tachyphylactic effect of DSCG is slowly reversed when cells previously treated with DSCG are placed in new medium. In the present experiments, the effect of washing the cells following pretreatment with cyproheptadine, promethazine, and DSCG was studied to determine the reversibility of their effects. Cells were sensitized and incubated either with or without drug solution for 5 minutes at 37°C. The cells were then washed twice in medium by suspension and centrifugation and finally resuspended and brought to 37°C. Then, they were challenged with antigen alone or simultaneously with drug. Histamine release from the cells which had been pretreated with drugs and washed twice before drug-antigen challenge was inhibited to the same extent as was the release from cells which had not been pre-incubated with the inhibitors ($p > 0.05$) (Fig. 8). This demonstrates that the tachyphylactic effect of DSCG can be removed by washing. The experiment also indicates that cyproheptadine and promethazine have little or no residual effect ($p > 0.05$).

In another series of tubes, the drug-treated cells, after two washes, were challenged with antigen alone. The only agent to produce a significant degree of inhibition under these conditions was cyproheptadine ($p < 0.05$). Nevertheless, a cumulative effect was not seen after washing and re-application of cyproheptadine ($p > 0.05$) (Fig. 8).

Fig. 8 Reversibility of drug response by washing. Isolated rat peritoneal cells were suspended in medium without or with drug (10 mcg/ml). After washing the cells twice in medium, the cells were resuspended and challenged with antigen with or without 10 mcg/ml of drug. Each bar represents the mean of 4 experiments ± SEM.


100
80
60
40
20
0
% INHIBITION
CHROMOGLYCATE
PROMETHAZINE
CYPROHEPTADINE
pretreat
challenge
medium
wash x 2
drug + Ag
drug
wash x 2
drug + Ag
drug
wash x 2
Ag

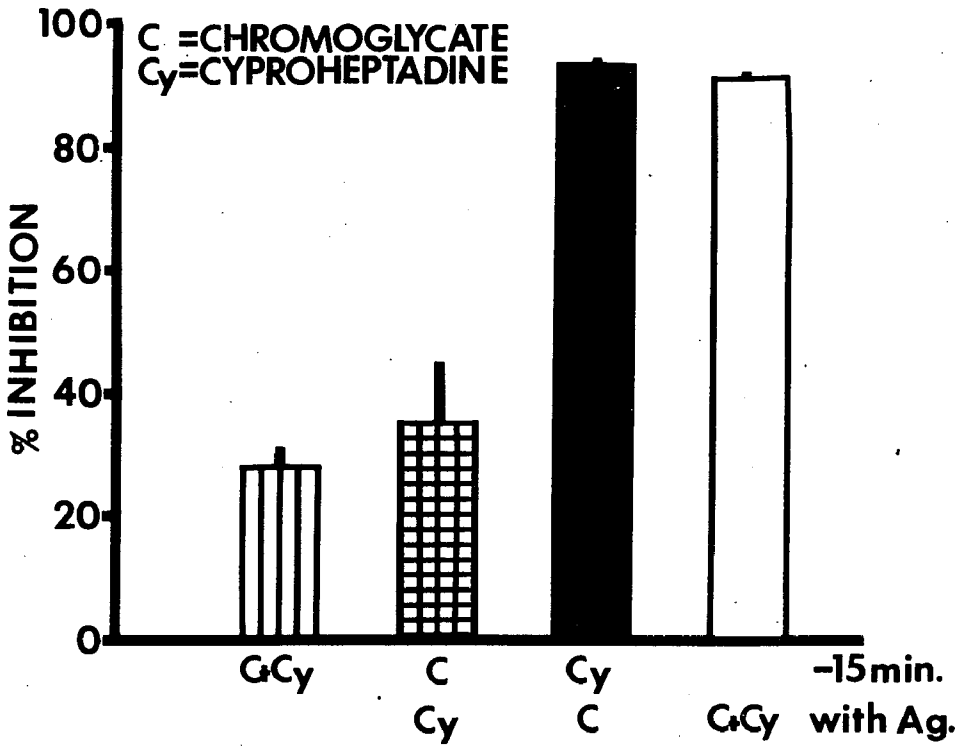
Interactions Between DSCG and Cyproheptadine

The demonstrated difference in the inhibitory effects of DSCG and cyproheptadine on the histamine release process indicated that the two drugs were acting by different mechanisms. This difference was studied in more detail by an evaluation of the effect of equal concentrations of DSCG and cyproheptadine (10 mcg/ml) on antigen-induced histamine release. The two drugs were administered either separately or together, 15 minutes prior to, or with antigen challenge.

It is seen in Figure 9 that, when both DSCG and cyproheptadine were added to the cells 15 minutes prior to antigen, inhibition of release was obtained equivalent to that of cyproheptadine alone (cf. cyproheptadine, Fig. 6). When DSCG was added to the cells 15 minutes prior to cyproheptadine and antigen, an inhibition of histamine release equivalent to cyproheptadine alone was again seen. However, when DSCG and antigen were added to a cell suspension previously or concurrently exposed to cyproheptadine, the inhibition of histamine release was approximately additive. Since the combined inhibitory effect was nearly 100%, a strictly additive effect could not be discerned from any synergism which might have occurred. These results demonstrate that DSCG in combination with cyproheptadine still produced tachyphylaxis but cross-tachyphylaxis to cyproheptadine did not occur.

Fig. 9 Independence of the inhibition of histamine release by DSCG and cyproheptadine: Passively sensitized peritoneal cells were treated with 10 mcg/ml each of DSCG and cyproheptadine using a latin square experimental design. The agents were administered 15 min before (-15 min) and with antigen challenge (with Ag). When no drug is indicated, medium alone was added to the cell suspension. Each bar represents the mean of four experiments ± SEM.

A statistical evaluation is presented in table 5.


C =CHROMOGLYCATE
Cy=CYPROHEPTADINE
% INHIBITION
100
80
60
40
20
0
GCy
C
Cy
Cy
C
GCy
-15min.
with Ag.

Interaction Between DSCG and Promethazine

An experiment identical to that described above for DSCG and cyproheptadine was carried out with DSCG and promethazine with essentially identical results (Fig. 10). DSCG, when added to the cell suspension 15 minutes before the simultaneous addition of promethazine and antigen, had no influence on the inhibitory effect of promethazine on histamine release (cf. Fig. 7 and Fig. 10); it was equivalent to that of promethazine alone. When DSCG was added to the cell suspension simultaneously with antigen, and promethazine was added either 15 minutes previously or together with DSCG and antigen, histamine release was inhibited nearly completely (Fig. 10). Thus, tachyphylaxis to DSCG still occurred, but no cross-tachyphylaxis with promethazine was produced. Statistical analysis of these interactions are given in Table 6.

The Effect of DSCG, Cyproheptadine and Promethazine on Histamine Release by Compound 48/80 and the Calcium Ionophore A23187

To extend the previous findings on the mechanism of action of the antihistamines and of DSCG, their effect on histamine release by the chemical releasing agents was investigated. Inhibition by DSCG of histamine release by Compound 48/80 and antigen occurred in the same concentration range, with some inhibition at 1.0 mcg/ml and maximum antagonism at 10.0 mcg/ml ($p < 0.05$) (Fig. 11). DSCG (3-30 mcg/ml)

Fig. 10 Independence of the inhibition of histamine release by DSCG and promethazine. Concentrations of DSCG and promethazine were 10 mcg/ml. Drugs were added to isolated rat peritoneal cell suspensions either together or separately with antigen challenge (with Ag) or 15 minutes before antigen (-15 min). When no drug is indicated, medium alone was added. Each bar represents the results of four experiments ± SEM.

The statistical evaluation is presented in table 6.

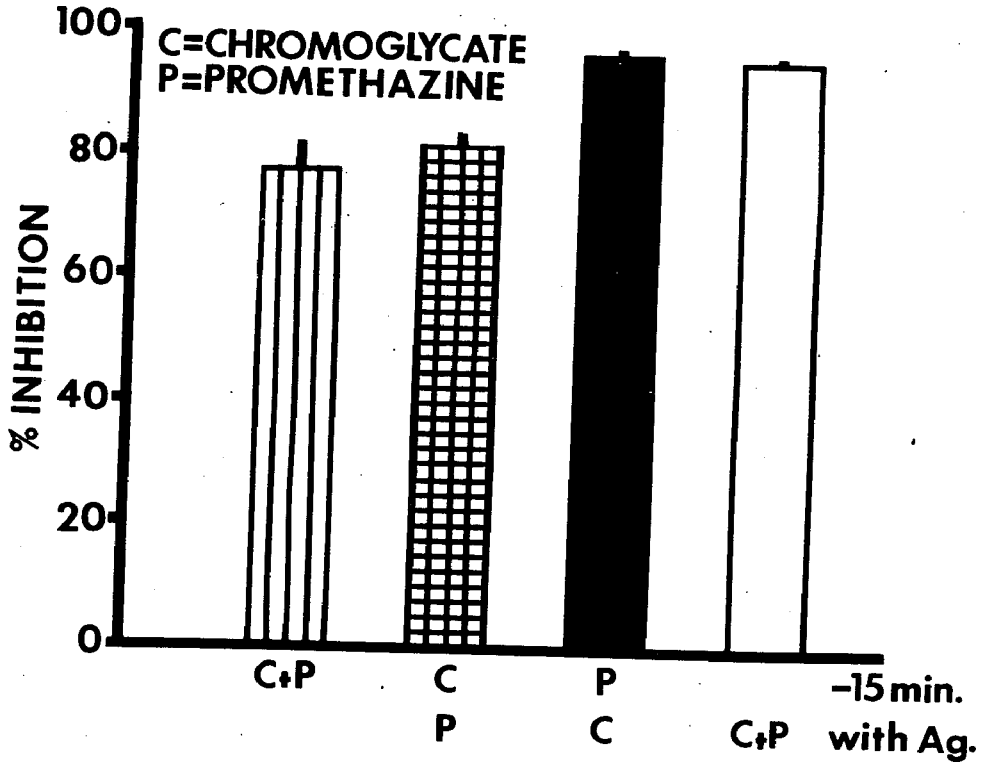
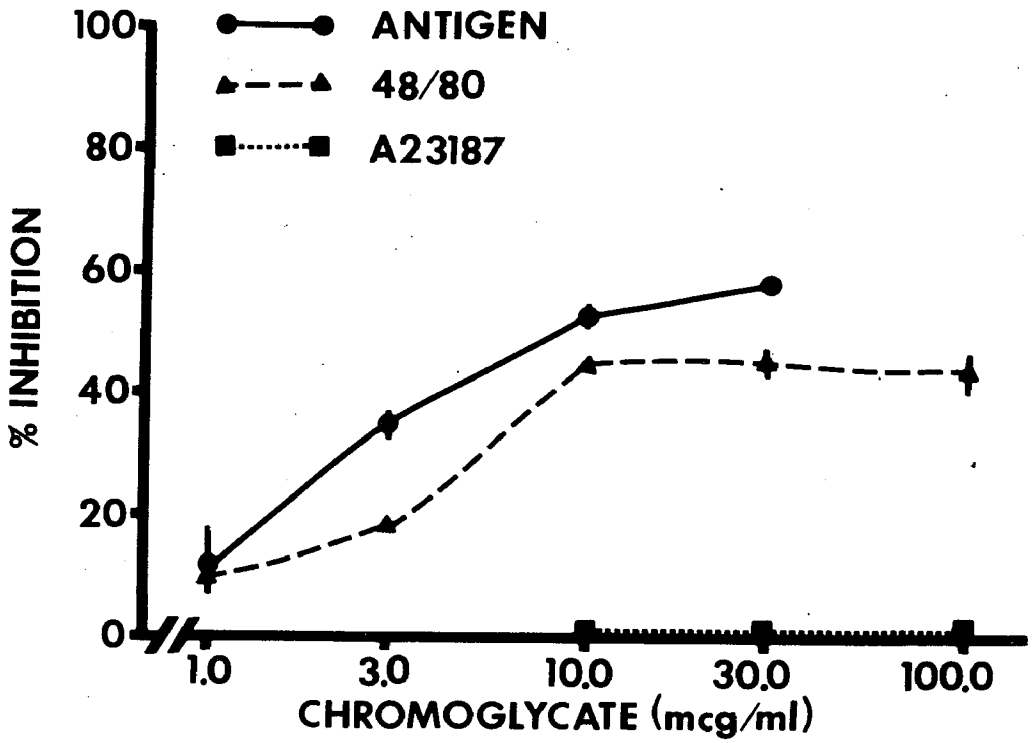

C=CHROMOGLYCATE
P=PROMETHAZINE
% INHIBITION
100
80
60
40
20
0
C+P
C
P
P
C
C+P
-15 min.
with Ag.

Fig. 11 Antagonism by DSCG of histamine release induced by antigen, Compound 48/80 and calcium ionophore A23187. Rat peritoneal cells were passively sensitized for antigen-induced release. Non-sensitized cells were used with 48/80 and A23187. DSCG was added simultaneously with antigen or the chemical releasing agents. Each point is the mean of 4 to 6 determinations. Vertical bars indicate ± SEM. Concentrations of 48/80 and A23187 were 0.5 mcg/ml.


100
80
60
40
20
0
% INHIBITION
ANTIGEN
48/80
A23187
1.0
3.0
10.0
30.0
100.0
CHROMOGLYCATE (mcg/ml)

had a greater efficacy as an antagonist of histamine released by antigen than Compound 48/80 ($p < 0.05$). DSCG did not antagonize histamine release by the calcium ionophore A23187 (Fig. 11).

When the cells were exposed to DSCG for 15 minutes prior to challenge, the decreased inhibition of 48/80-induced release occurred as was observed with antigen challenge ($p < 0.05$) (Fig. 12). Pretreatment with DSCG was again completely ineffective in antagonizing histamine release by the calcium ionophore A23187 (Fig. 12).

Cyproheptadine was also found to antagonize antigen and Compound 48/80 induced histamine release with the same efficacy and over approximately the same concentration range; that is, 1 to 10 mcg/ml ($p > 0.05$) (Fig. 13). At 10 mcg/ml, the antagonism by cyproheptadine of ionophore histamine release was quite variable and ranged from augmentation to about 25% inhibition, thus accounting for the large standard error. As the concentration of cyproheptadine was increased, 3 to 10-fold, the inhibition became more marked and less variable. The much higher concentration of cyproheptadine required to inhibit ionophore-induced release indicates that this antagonism differs from the inhibition of release induced by antigen or 48/80. At a concentration of 10 mcg/ml, cyproheptadine was as effective when administered 15 minutes before as when administered with any of the releasing agents ($p > 0.05$) (Fig. 14).

Fig. 12 Effect of pretreatment time on DSCG antagonism of histamine release by antigen, Compound 48/80 and calcium ionophore A23187. Cells were treated with 10 mcg/ml of DSCG either 15 minutes before or at the time of challenge. Non-sensitized cells were used with 48/80 and A23187. Each bar represents the mean of 4 to 6 determinations ± SEM.

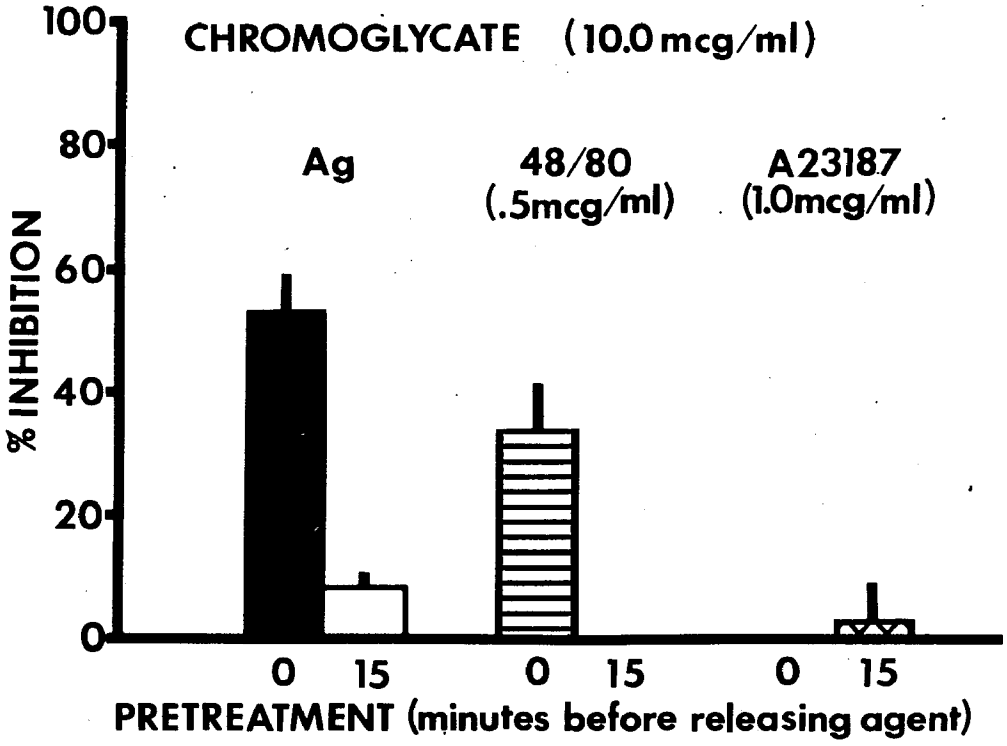

CHROMOGLYCATE (10.0 mcg/ml)
Ag
48/80 (.5mcg/ml)
A23187 (1.0mcg/ml)
% INHIBITION
100
80
60
40
20
0
0 15 0 15 0 15
PRETREATMENT (minutes before releasing agent)

Fig. 13 Antagonism by cyproheptadine of histamine release induced by antigen, Compound 48/80 and calcium ionophore A23187. Rat peritoneal cells were passively sensitized for antigen-induced release. Non-sensitized cells were used with 48/80 and A23187. Cyproheptadine was added simultaneously with antigen or the chemical releasing agents. Each point is the mean of 4 to 6 experiments ± SEM. Concentrations of 48/80 and A23187 were 0.5 mcg/ml.

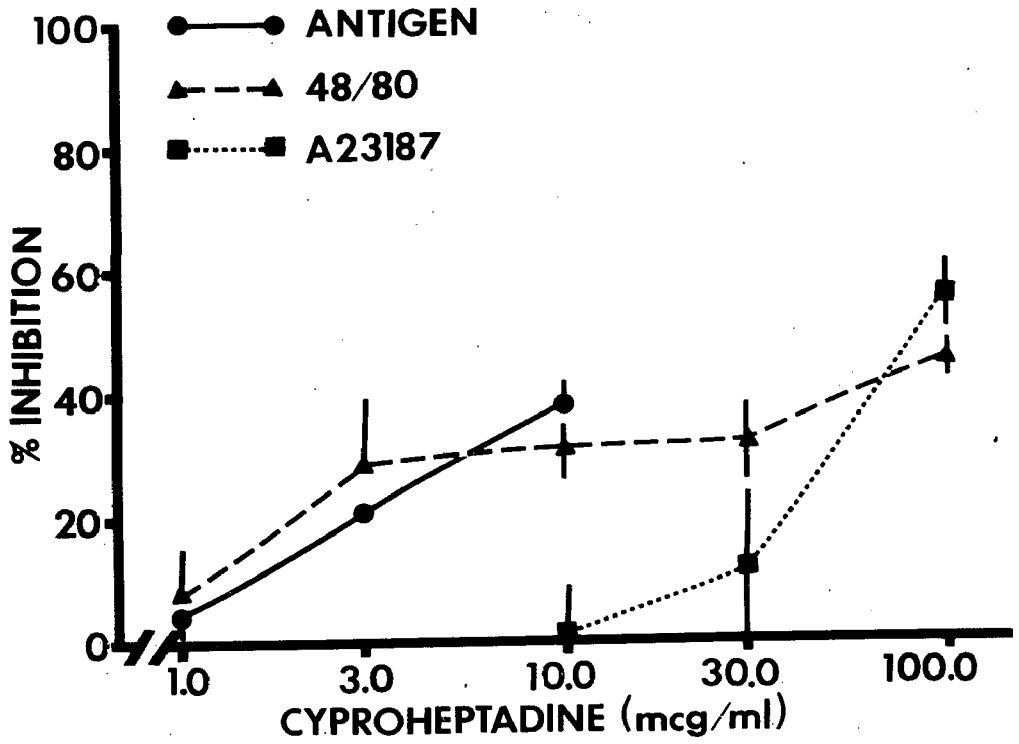
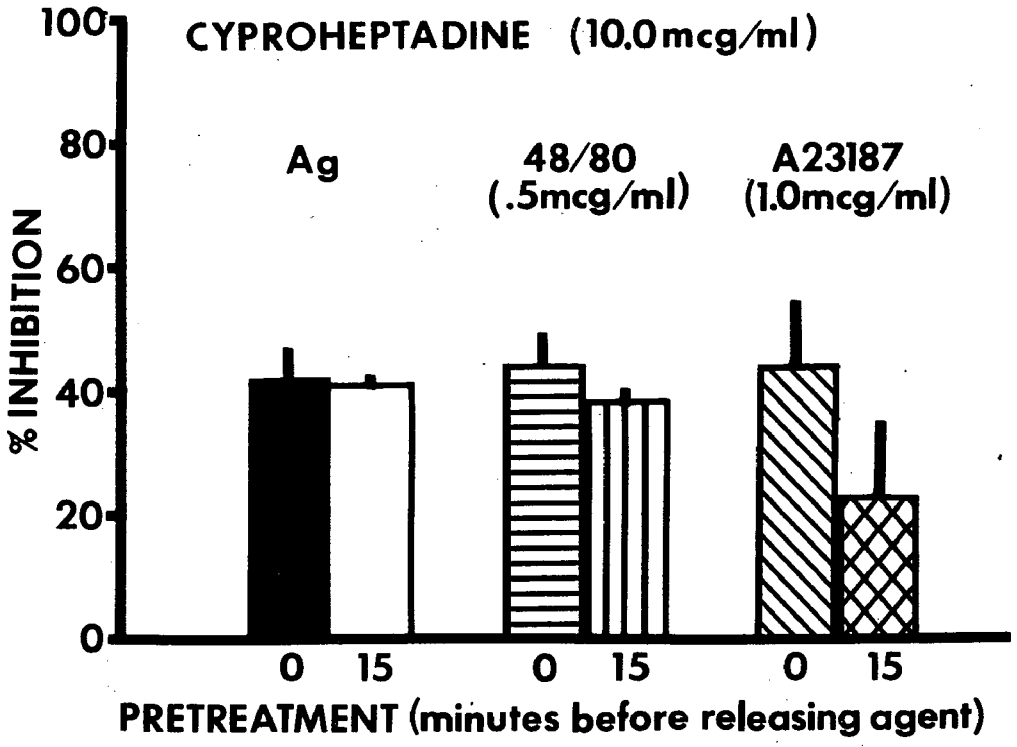

ANTIGEN
48/80
A23187
100
80
60
40
20
0
% INHIBITION
1.0
3.0
10.0
30.0
100.0
CYPROHEPTADINE (mcg/ml)

Fig. 14 Effect of cyproheptadine pretreatment time on histamine release from isolated rat peritoneal cells by antigen, Compound 48/80 and calcium ionophore A23187. Non-sensitized cells were used with 48/80 and the ionophore. Each bar represents the mean of 4 to 6 determinations ± SEM. No statistically significant differences exist among any pairs of means.


CYPROHEPTADINE (10.0mcg/ml)
Ag
48/80
(.5mcg/ml)
A23187
(1.0mcg/ml)
% INHIBITION
100
80
60
40
20
0
0
15
0
15
0
15
PRETREATMENT (minutes before releasing agent)

Dose-response curves indicate that a lower concentration of promethazine, when added at the time of challenge, was necessary to antagonize antigen-induced histamine release from peritoneal cells than the concentration required to inhibit release induced by either of the chemical mediators ($p < 0.05$) (Fig. 15).

When promethazine was added to the cell suspension 15 minutes before challenge, the decrease in inhibitory effect with time, which had been shown in a previous experiment for antigen-induced release, also was observed with Compound 48/80 and calcium ionophore-induced release ($p < 0.05$). The decrement appeared to be more prominent with these latter agents than when antigen was used (Fig. 16).

Effect of Specific Histamine Receptor Agonist and Antagonist on Antigen-Induced Histamine Release

All of the previous findings indicate that receptors for the antihistamines and DSCG which antagonize histamine release from the sensitized rat peritoneal cells are different. Since DSCG does not antagonize the effect of histamine at the H_1 receptor, the antihistamines were studied to determine whether they exerted their effect on the rat mast cell through one of the currently recognized receptors for histamine. Black et al. (29) have demonstrated that different effects of histamine can be inhibited selectively by different blocking agents, and classified these effects as being affected by either H_1- or H_2-receptors (cf. Table 3). In the basophil, exogenous histamine in-

Fig. 15 Antagonism by promethazine of histamine release from rat isolated peritoneal cells induced by antigen, Compound 48/80 (0.5 mcg/ml) or calcium ionophore A23187 (0.5 mcg/ml). Promethazine was added to the cell suspension at the time of addition of antigen or the chemical releasing agents. Non-sensitized cells were used with 48/80 or the ionophore. Each point represents the mean of 4 to 6 experiments ± SEM.

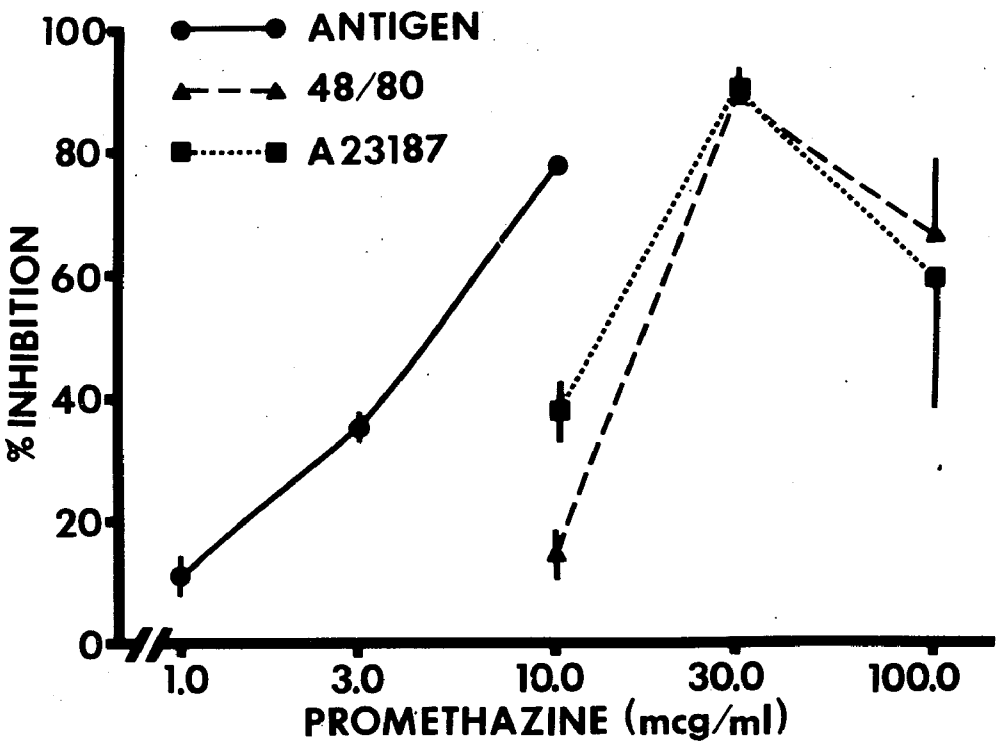
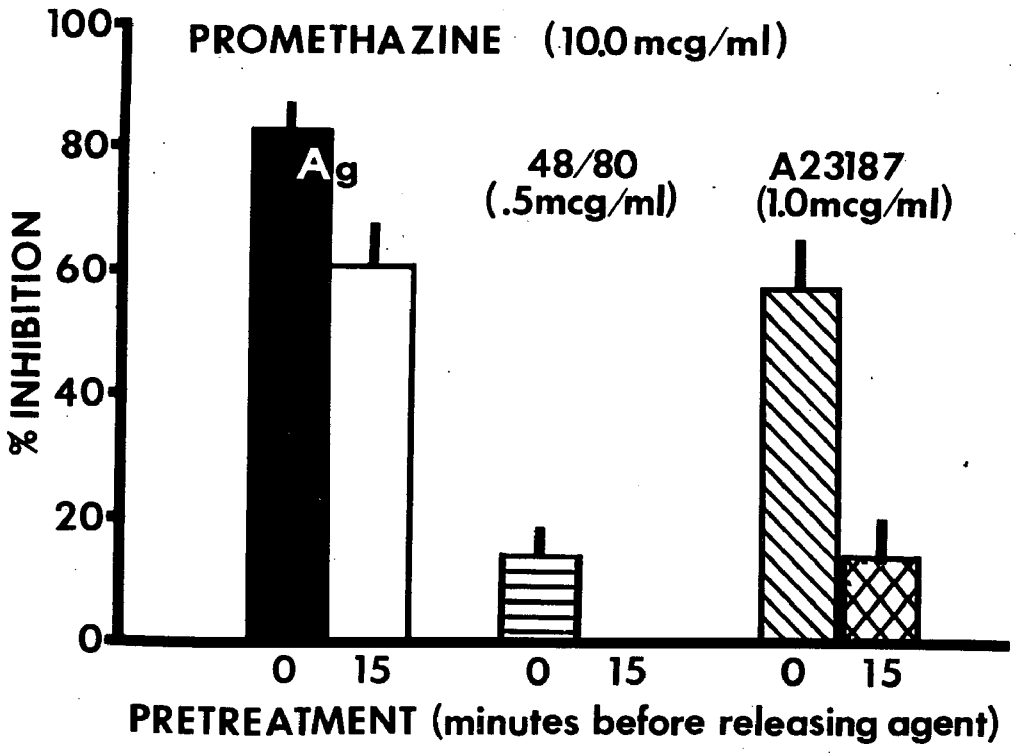

ANTIGEN
48/80
A23187
% INHIBITION
100
80
60
40
20
0
1.0
3.0
10.0
30.0
100.0
PROMETHAZINE (mcg/ml)

Fig. 16 Effect of promethazine pretreatment time on histamine release from isolated rat peritoneal cells induced by antigen, 48/80 or calcium ionophore A23187. Non-sensitized cells were used with 48/80 and the ionophore. Each bar represents the mean of 4 to 6 determinations ± SEM.

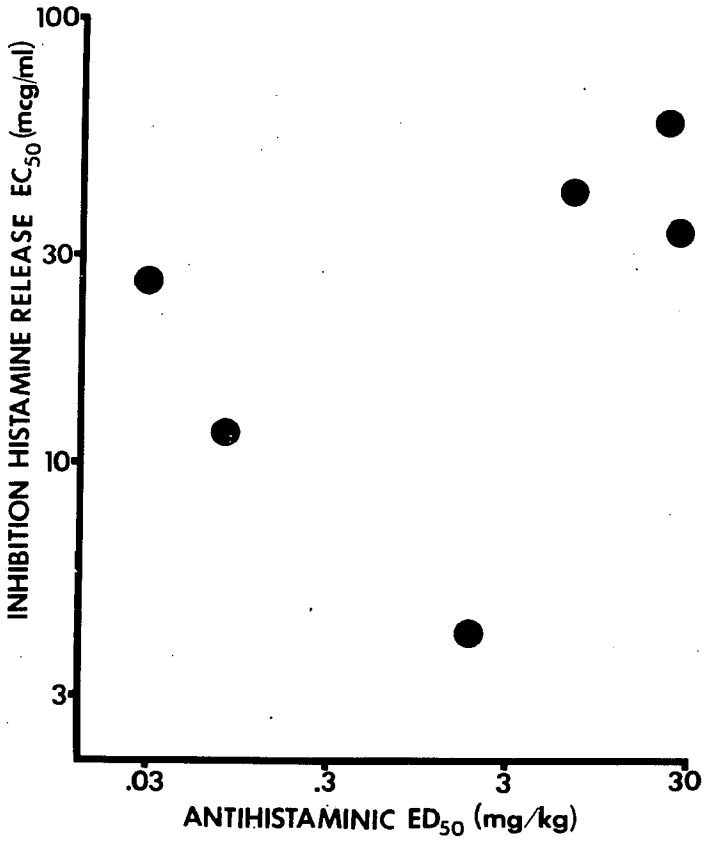

PROMETHAZINE (10.0 mcg/ml)
100
80
60
40
20
0
% INHIBITION
Ag
48/80
(.5mcg/ml)
A23187
(1.0mcg/ml)
0
15
0
15
0
15
PRETREATMENT (minutes before releasing agent)

hibits histamine release; however, this antagonism could be blocked by H_2-antihistamines. The effect of these agents was studied to determine whether they antagonized the action of exogenous histamine, including that released spontaneously and the histamine released initially by the mediators on the H_1-receptor.

Comparison of H_1-Antihistaminic Potency and Antagonism of Histamine Release

All of the H_1-antihistaminic agents tested in the preliminary experiments antagonized antigen-induced histamine release if the concentration was sufficient (Table 4). To determine whether there was a relationship between the H_1-antihistaminic potency and the ability to antagonize histamine release, the oral dose of the drug necessary to protect guinea pigs from the lethal effects of histamine aerosols was found. Figure 17 demonstrates the relationship between the potency of the H_1-antagonists to inhibit half of the histamine released by antigen (EC_{50}) and to protect half of the guinea pigs tested from the lethal effect of a 2% histamine diphosphate aerosol. There was approximately a 1000-fold potency range for these agents as antagonists of bronchospasm - with chlorpheniramine being the most potent and diphenhydramine the least potent. The relative potency of the compounds in this system were comparable to <u>in vitro</u> H_1-antihistaminic potency in the literature (15-17,291) and neither DSCG nor metiamide inhibited bronchospasm (data not shown). The relative

Fig. 17 Relative potency of different chemical categories of antihistamines as antagonists of histamine aerosol lethality in guinea pigs and antigen-induced histamine release. ED_{50} dose of antihistamine required to protect half of the guinea pigs from the lethal effect of a histamine aerosol for 5 minutes. EC_{50} is the concentration of agent required to antagonize half of the antigen-induced histamine release from sensitized rat peritoneal cells. No statistically significant correlation was found by regression analysis ($p < 0.70$).


100
30
10
3
INHIBITION HISTAMINE RELEASE EC_{50} (mcg/ml)
.03
.3
3
30
ANTIHISTAMINIC ED_{50} (mg/kg)

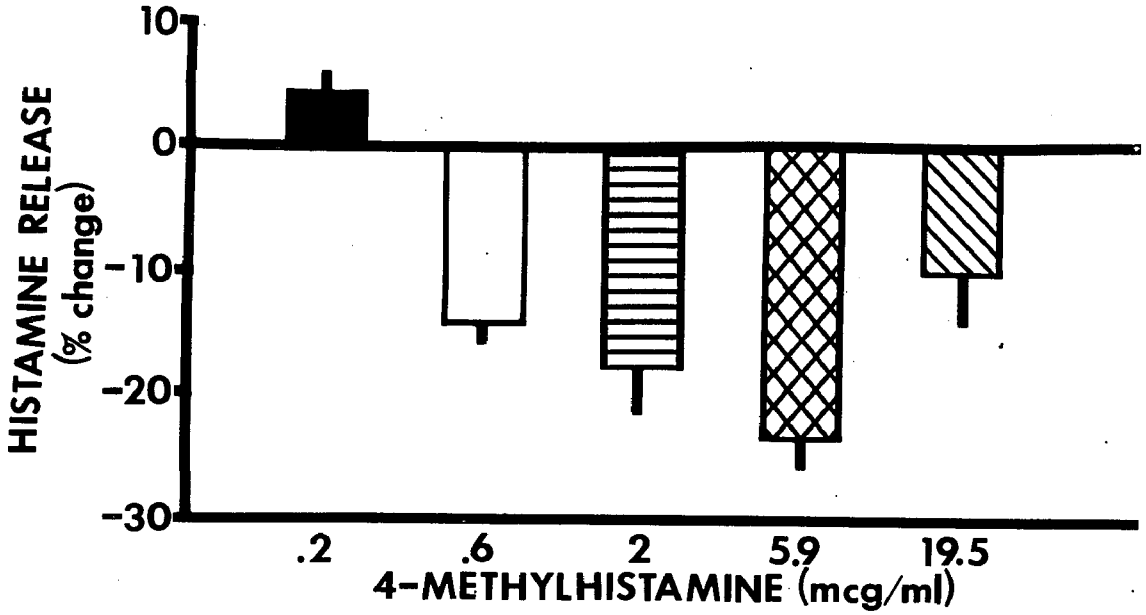
potency of the compound to reduce antigen-induced histamine release occurred over a 12-fold range -- promethazine was the most potent and cyclizine the least -- in antagonizing half of the histamine release induced by antigen (EC_{50}). No correlation was found between the ability of these agents to antagonize these parameters by 50% ($p < 0.70$) (Fig. 17). The lack of correlation indicates that either the H_1-receptors on the mast cell are different from those in smooth muscle, or that these compounds act through another mechanism.

In Vitro Mast Cell Histamine Receptor Evaluation

In preliminary experiments, it was found that extracellular histamine did not significantly affect antigen-induced histamine release. If rat mast cells contain both H_1- and H_2-receptors of equal sensitivity in any particular experiment, no influence on histamine release by histamine itself would be expected. Therefore, it was necessary to employ selective receptor agonists and antagonists. The compound, 4-methylhistamine, has been shown to be 170 times more potent on H_2-receptors whereas 2-pyridineethaneamine is 30 times more active on H_1- than on H_2-receptors (67).

In the concentrations of 0.6 to 5.9 mcg/ml, 4-methylhistamine caused a maximum inhibition of antigen-induced histamine release from peritoneal cells of approximately 20% ($p < 0.05$) (Fig. 18). Based on

Fig. 18 Effect of 4-methylhistamine on antigen-induced histamine release from passively sensitized rat peritoneal cells in vitro. Each bar represents the mean of 4 to 8 experiments ± SEM.


10
0
-10
-20
-30
HISTAMINE RELEASE
(% change)
.2
.6
2
5.9
19.5
4-METHYLHISTAMINE (mcg/ml)

the relative potency in stimulating gastric secretion in rats, which is a specific H_2 histamine system, the concentrations of 4-methylhistamine used were equivalent to from .08 to 7.6 mcg/ml of histamine (67). This is equivalent to the range of extracellular histamine levels found in vitro following antigen-induced release in the present experiments. If cyproheptadine and promethazine specifically inhibited H_1-receptors on mast cells, histamine released by antigen might activate preferentially H_2-receptors and inhibit further release. In order to test this concept further, a specific H_2-antagonist, metiamide, was employed. If the rat mast cell has H_1- and H_2-receptors, metiamide would be expected to augment release as well as block the effect of 4-methylhistamine.

The effect of metiamide, at 1-10 mcg/ml, has been shown to be an effective antagonist of stimulation of the H_2-receptors by histamine in the sensitized basophil (170,174). However, when added to sensitized peritoneal cells, the effect of metiamide (1.0 mcg/ml) on antigen-induced histamine release was slight and inconsistent ($p > 0.05$) (Figs. 19 and 20). As demonstrated in Figure 20, although antigen-induced histamine release is consistently inhibited by 4-methylhistamine, this effect is not blocked by metiamide ($p > 0.05$).

To determine more directly if an H_1-receptor played a role in the inhibition of histamine release by cyproheptadine and promethazine, the effect of the specific H_1-stimulant, 2-pyridineethanamine, was

Fig. 19 Effect of metiamide on antigen-induced histamine release from passively sensitized rat peritoneal cells. Each bar represents 7 experiments ± SEM.

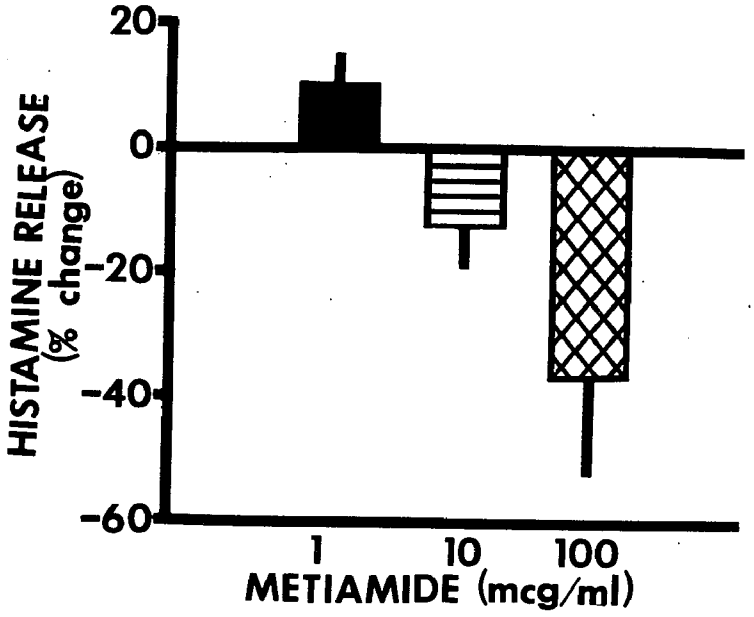
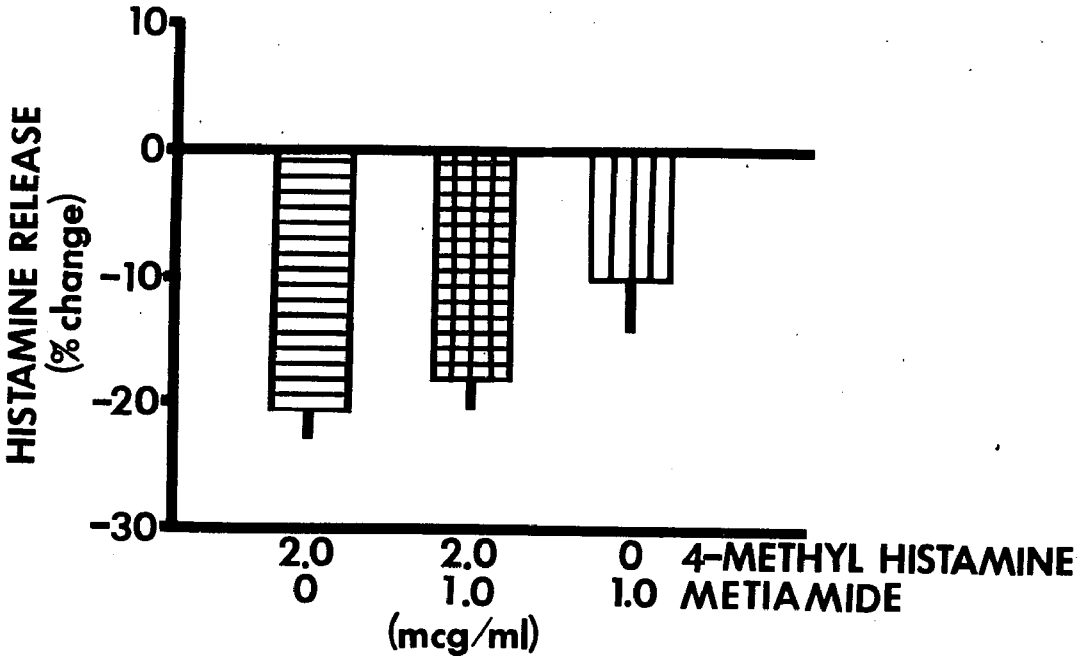

20
0
-20
-40
-60
HISTAMINE RELEASE
(% change)
1
10
100
METIAMIDE (mcg/ml)

Fig. 20 Effect of 4-methylhistamine and metiamide on antigen-induced histamine release from sensitized rat peritoneal cells. Each bar represents the mean of 12 experiments ± SEM.


10
0
-10
-20
-30
HISTAMINE RELEASE
(% change)
2.0
0
2.0
1.0
0
1.0
4-METHYL HISTAMINE
METIAMIDE
(mcg/ml)

studied on antigen-induced histamine release. The concentrations of 2-pyridineethanamine used in the experiments (Fig. 21) was calculated to be equivalent to about 0.3 to 3.3 mcg/ml of histamine based on relative potency studies in guinea pig ileum (67). At these concentrations, the H_1-histamine receptor stimulant did not cause a significant augmentation of antigen-induced histamine release ($p > 0.05$), suggesting that an H_1-receptor on rat mast cells is not significant in antagonism of antigen-induced histamine release.

A Comparison of Local Anesthetic Activity and Antagonism of Antigen-Induced Histamine Release

Antihistamines can produce a local anesthetic effect (163) and, thus, may inhibit histamine release by stabilizing the membrane. The activity of these agents as local anesthetics was, therefore, compared to their ability to antagonize antigen-induced histamine release (Fig. 22). The local anesthetic activity of the agents, as assessed by the method of Bulbring and Wadja (39) on the guinea pig back, did not correlate with their ability to inhibit antigen-induced histamine release from rat peritoneal cells ($p = 0.52$).

Fig. 21 The effect of 2-pyridineethanamine on antigen-induced histamine release from rat peritoneal cells. Each bar represents 8 experiments ± SEM.

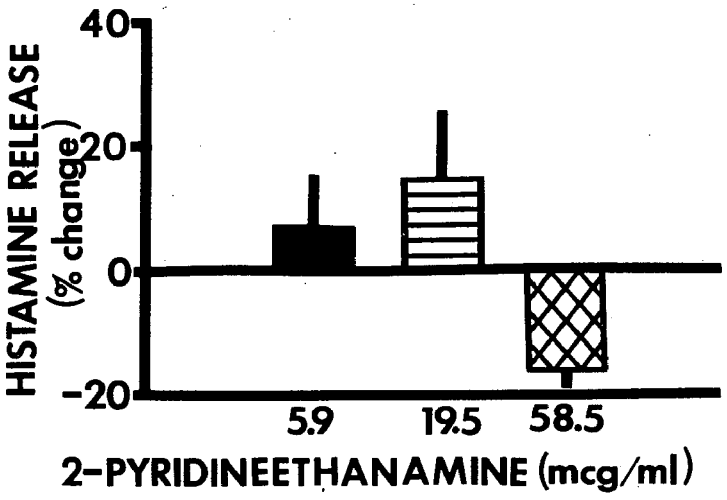
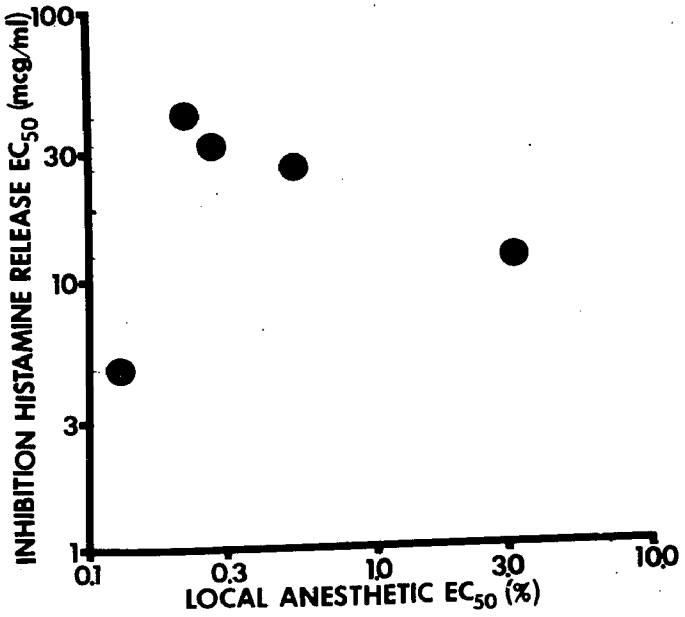

40
20
0
-20
HISTAMINE RELEASE
(% change)
5.9
19.5
58.5
2-PYRIDINEETHANAMINE (mcg/ml)

Fig. 22 The relative potency of representative antihistamines of different chemical categories as antagonists of antigen-induced histamine release and local anesthetic effect. EC_{50} concentration decreased response by 50%. Concentration of drug injected intradermally into guinea pig back that blocked the response to half of the pain stimuli caused by a pin prick. Concentration of drug that decreased antigen-induced histamine release by 50%. Regression analysis correlation was statistically insignificant ($p = 0.52$).


INHIBITION HISTAMINE RELEASE EC_{50} (mcg/ml)
100
30
10
3
1
0.1
0.3
1.0
3.0
10.0
LOCAL ANESTHETIC EC_{50} (%)

DISCUSSION

Mota and DaSilva (207) demonstrated that H_1-antihistamines would antagonize antigen-induced histamine release from mast cells. However, the only system they used, which probably contained IgE antibodies, required 0.25 - 1.0 mg/ml of the H_1-antihistamine to inhibit release. At these concentrations, many antihistamines will induce histamine release (5,306). Promethazine and cyproheptadine have also been reported to antagonize histamine release by Compound 48/80 at about 10 mcg/ml. However, this brief communication did not describe the inhibition (306). A more detailed report of the effect of antihistamines as antagonists of histamine release is that of Lichtenstein and Gillespie in the human basophil (170). The H_1-antihistamines' ability to inhibit histamine release from both the basophil and mast cell is extremely interesting from the point of view of the mechanics of inhibition of release, since DSCG antagonizes histamine release only in the mast cell (7). The present study was undertaken to examine the mechanisms of action of DSCG, cyproheptadine, and promethazine as antagonists of histamine release in an effort to determine whether these antihistamines might have a mechanism different from that of DSCG.

Antagonism of antigen-induced histamine release by DSCG is of extremely short duration in the rat mast cell. Cyproheptadine and

promethazine were found to have a sustained effect when administered up to one hour before antigen challenge. DSCG not only was ineffective when administered 15 minutes prior to antigen challenge but, at a sufficient concentration, DSCG also caused tachyphylaxis; that is, it blocked the inhibitory effect of the drug in a subsequent DSCG-antigen exposure of the cells. Since both the rapid loss of the inhibitory effect of DSCG and tachyphylaxis induced with DSCG are temporally identical (160), these two effects of pretreatment are probably the result of the same mechanism. The complete removal of the inhibitory effect of promethazine and the tachyphylactic effect of DSCG from the cells by washing indicates that these compounds do not bind tightly to effector sites. The binding of cyproheptadine to receptors appears to be somewhat less labile than for the other two agents since its effect is only partially reversed by washing. In contrast to DSCG, a 15-minute exposure to cyproheptadine or promethazine prior to challenge did not decrease the inhibition when the same agent was administered along with antigen challenge. These results suggested a different mechanism of blockage by promethazine and cyproheptadine from that by DSCG. This possibility was supported by two additional facts: 1) when the cells were in a state of tachyphylaxis to DSCG, both promethazine and cyproheptadine still blocked release and 2) DSCG, administered with antigen, provided additional blockade to that established by the two antihistamines administered

prior to or with challenge.

The effect of DSCG, cyproheptadine, and promethazine on histamine release by antigen, Compound 48/80 and the calcium ionophore A23187 were studied. In preliminary experiments, concentrations of Compound 48/80 and A23187 were selected which did not cause any observable toxic effect on the cells as determined by Trypan Blue exclusion, and that released about the same proportion of histamine from mast cells as the antigen/antibody reaction. The three antagonists were approximately equipotent inhibitors of antigen-induced histamine release, the dose-response curves encompassing the concentration range of 1 to 10 mcg/ml.

Cyproheptadine and DSCG were maximally effective antagonists of the histamine release induced by 48/80 at a concentration of 10 mcg/ml. Promethazine only caused a very slight degree of inhibition of 48/80-induced release at this concentration, however, and 30 mcg/ml of this antihistamine was required to produce a high level of inhibition of release by this agonist. The apparent decrease in antagonism of histamine release at 100 mcg/ml of promethazine is probably due to the induction of histamine release by the antihistamine (5,170). Since all three antagonists block antigen-induced release effectively at the same concentration, but promethazine is required in a higher concentration to inhibit 48/80-induced release, cyproheptadine and DSCG probably inhibit at a site different from the one antagonized by promethazine.

Histamine release by the ionophore was not inhibited by DSCG, was only inhibited by high concentrations of promethazine, and at even higher concentrations of cyproheptadine. The higher concentrations of the antihistamines required to inhibit A23187-induced histamine release than antigen-induced release would indicate some additional mechanism appearing at these concentrations.

Each of the three antagonists produced a different spectrum of inhibition of the histamine released by the three agonists. There are three possible sites where this antagonism could occur - extracellular, intracellular, and at the cell membrane. The three releasing agents have the same requirements for extracellular components, that is, energy and calcium. The inability of the inhibitors to antagonize A23187-induced histamine release at the lower concentration suggests that they were not affecting the necessary components of the release process in the extracellular fluid since these components are common to all three methods of releasing histamine from mast cells (55,87, 138,224).

Incubation of sensitized cells with DSCG and then addition of antigen incubated with DSCG results in histamine release equivalent to the release without the chromone in either step. These findings indicate DSCG does not exert its antagonism through an interaction with antigen, antibody or antibody receptor. The antagonism of release by cyproheptadine has the same relative potency and efficacy as well

as the same prolonged blockade for both antigen/antibody and 48/80-induced histamine release. This would indicate that the compound inhibits at a site common to both types of release. Since 48/80-induced release does not involve an immune mechanism, inhibition of antigen/antibody release by cyproheptadine also cannot be due simply to the reaction with the immune components.

The ineffectiveness of DSCG, and the higher concentrations of the antihistamine required to decrease A23187 induced histamine release, as compared to the level necessary to inhibit the antigen/antibody reaction, indicate that these compounds do not antagonize a step in the release process subsequent to the calcium influx.

Serine esterase inhibitors abolish histamine release induced by antigen, 48/80, or A23187 (58). If DSCG or the antihistamines antagonized serine esterase, they should also inhibit histamine release by the calcium ionophore; however, the three agents either did not inhibit release by A23187 or did so only at a higher concentration than required for antigen-induced release.

The three releasing agents used in the present experiments all require a source of energy and calcium. Therefore, since these agents either do not inhibit histamine release by the calcium ionophore, or do so only at much higher concentrations, it seems improbable that they inhibit either the calcium or energy requiring step of histamine release. The ionophores can affect other cell systems such as energy formation (231); however, this does not appear to be important in

histamine release from the mast cell. If it were a problem, antagonism of release instead of initiation would be expected, and secondly, the release of histamine is so rapid that most of the energy required has to be present in the cell at the time of challenge.

Although DSCG has been reported to act as a phosphodiesterase antagonist (243), this seems improbable. The phosphodiesterase inhibitor, theophylline and stimulants of adenylate cyclase are synergistic in increasing c-AMP levels, and the inhibiting histamine release (102), however DSCG does not share this characteristic with this xanthine (156). In preliminary experiments (data not presented), theophylline alone did not have a consistent effect on antigen-induced histamine release even at very high concentrations. This is in contrast to the effect of DSCG or the antihistamines which consistently inhibited histamine release even at relatively low concentrations. Increases in c-AMP does inhibit histamine release in the mast cell (167,175). Little is known about the events that occur between activation of the cell surface by the antigen/antibody reaction and the events that occur inside the cell that result in histamine release.

H_1-antihistamines have local anesthetic properties (54,163,176) and some local anesthetics have been shown to inhibit histamine release induced by antigen (136,234) or A23187 (234). The local anesthetics may (146) or may not (136) antagonize 48/80-induced histamine release. In contrast to DSCG or the antihistamines, the local anesthetics were more effective when incubated with cells before challenge, and the effect of the

local anesthetics could not be removed by washing (146). The tenacity of the local anesthetics and cyproheptadine for the cells was a common characteristic. However, while cyproheptadine is a much more potent antagonist of histamine release than tetracaine, it is a much weaker local anesthetic (236, data not presented). The only characteristic which may be shared at equivalent concentrations by the local anesthetics and antihistamines on the mast cell is the inhibition of 48/80 (146) and A23187 (234) induced release by the lowest effective concentrations of tetracaine, and the highest concentrations of promethazine before the antihistamine begins to potentiate histamine release. This may indicate a secondary inhibition of histamine release by higher concentration of antihistamines. At lower concentrations (10mcg/ml) the H_1-antagonists and DSCG probably antagonize antigen-induced histamine release by a mechanism that is not related to a local anesthetic type of membrane stabilization.

The effect of a series of antihistamines was studied to determine whether the potency of these agents in inhibiting histamine release could be related to a general membrane stabilization similar to that caused by local anesthetics. In the present experiments, there was no correlation between the effect of the H_1-antagonists as local anesthetics and their ability to inhibit histamine release. Therefore, it is improbable that this effect is related to a local anesthetic type of membrane stabilization. Although this limits the antagonism to the cell membrane, it does not help define the receptor in the

context of those known to be acted on by the H_1-antihistamines.

In the investigation of receptor types, there was no correlation between the antagonism of antigen-induced histamine release and the effect of histamine on guinea pig bronchial smooth muscle. This demonstrated that the antihistamines did not antagonize a receptor in the rat peritoneal cell identical to the one in bronchial smooth muscle. The relative potency as antagonists of histamine release by the agents used in the present experiments was of the same order as that reported by Lichtenstein and Gillespie in the basophil and they stated that there was no correlation between the antagonism of release by the H_1-receptor antagonists and the ability of these agents to antagonize the effect of histamine on the guinea pig ileum (170).

Since inhibition of histamine release from basophils by exogenous histamine can be blocked by H_2-histamine receptor blocking agents, and this effect is additive to that of the H_1-antagonists, the possibility of the action of the antihistamines on a histamine feedback mechanism was examined. In the present study of the rat mast cells, the H_2-agonist, 4-methylhistamine, antagonized histamine release by a maximum of 20%, much less than the 80-90% inhibition of antigen-induced histamine release produced by exogenous histamine in the basophil (35). To determine whether the inhibition of histamine release by 4-methylhistamine was indeed an H_2-stimulant effect, the response to 4-methylhistamine on antigen-induced histamine release

was examined in the presence and absence of metiamide. Metiamide alone did not affect histamine release in the concentrations used, nor did it modify the antagonism by 4-methylhistamine, which suggests that the effect of the latter was not due to H_2-receptor stimulation of the rat mast cell. Lack of a consistent augmentation of histamine release by the selective H_1-receptor agonist, 2-pyridineethanamine, additionally suggested that the mast cell does not have H_1-receptors. Thus, the antagonism of histamine release by promethazine and cyproheptadine does not result from an action on conventional histamine receptors.

The results discussed above indicate that cyproheptadine, DSCG and promethazine do not antagonize any steps of the histamine release process subsequent to the calcium influx. The antigen/antibody reaction and 48/80 both activate histamine release through a cell surface mechanism (101,119); therefore, it would be reasonable to conclude that DSCG, cyproheptadine, and promethazine antagonize histamine release by an action at the cell membrane.

Foreman et al. (84) demonstrated that DSCG antagonizes calcium influx into the cell. The present experiments demonstrate that the antihistamines also inhibit histamine release unless the cellular membrane steps are bypassed with the calcium ionophore. This indicates that the antihistamines also inhibit by stopping the calcium influx. Support for this idea also comes from the fact that the antihistamines, as DSCG, do not affect spontaneous release which is calcium independent (86).

There are currently no published reports that the increase in mast cell membrane permeability to calcium initiated by the antigen-antibody reaction that results in histamine release may be a multi-step process that can be antagonized at more than one stage. The difference in the antagonistic characteristics of DSCG and the antihistamines, however, indicate that there are at least three steps in the cell membrane. The first step is induced by the antigen/antibody reaction and is blocked by all three agents at low concentrations. The second step is induced by Compound 48/80 and blocked only by DSCG and cyproheptadine. The third step is the final one which allows calcium influx and is not blocked by any of the three antagonists studied. When mast cells or basophils are challenged in the cold (85,135,172) or in the absence of calcium (76,81,169) so that histamine release does not occur, the cells are desensitized so that they do not release histamine on subsequent challenge. Antigen will desensitize cells which have the specific antibody on the surface to itself but not to Compound 48/80, while Compound 48/80 can desensitize cells to both itself and antigen. However, neither of these agonists will desensitize mast cells to histamine release induced by A23187 (54,81,136,257). This would indicate a sequence of steps, and the selective antagonism of the histamine released by DSCG, and the antihistamines help define the order in which these steps occur.

It can be concluded that these studies have demonstrated a mode of antagonism of histamine release distinct from that of DSCG. Both

methods of antagonizing histamine release are associated with a membrane phenomenon. This would indicate that alteration of the membrane producing a calcium influx which results in histamine release is not a direct perturbation of the membrane but rather a more involved process having two or more phases. The antigen/antibody reaction, Compound 48/80, and the calcium ionophore A23187 can cause histamine release at several different points within the cell membrane, separated one from another by selective blockade either of the site, or between sites, with promethazine, DSCG, or cyproheptadine.

Identification of two or more pharmacologically inhibitable steps, which are not related to other activities of the antagonists, open the potential for identifying chemicals which will selectively block these steps.

SUMMARY

The characterization of IgE and its relationship to allergic mediator release was the cornerstone on which the prevailing study of allergy is based. The quoin that initiated the pharmacological interest in allergy was the discovery of DSCG.

Antigen-induced histamine release from sensitized rat peritoneal cells is inhibited by DSCG and the H_1-antihistamines. The allergic release of histamine from the basophil has been reported to be antagonized by the H_1-antihistamines, but not DSCG. The present study was designed to study how the H_1-antihistamines antagonize histamine release from rat peritoneal cells, and to determine whether this is by the same, or a different, mechanism from that of DSCG. Several chemical categories of antihistamines were studied and a detailed comparison was made of the antagonism by DSCG, promethazine and cyproheptadine of histamine release from rat peritoneal cells by antigen, Compound 48/80 or the calcium ionophore A23187.

All of the H_1-antihistamines studied antagonized histamine release from passively sensitized rat peritoneal cells. In contrast to the decreasing inhibition following preincubation of cells with DSCG and the tachyphylactic effect produced by DSCG, the antagonism of release by the H_1-antagonists persisted as long as the antihistamines were in

contact with the cells. The inhibition of histamine release by promethazine and the tachyphylactic effect of DSCG could be removed by washing; however, the antagonism of release by cyproheptadine persisted even after the cells were washed. When cells were in a state of tachyphylaxis to DSCG, release could still be inhibited by the antihistamines, and no interaction between the antagonistic effects of these agents was observed.

Antigen-induced histamine release was maximally antagonized by DSCG, cyproheptadine, and promethazine at 10 mcg/ml; however, they were relatively ineffective inhibitors of release by A23187 at this concentration. At this level, 48/80-induced release was antagonized by DSCG and cyproheptadine but not by promethazine. Pretreatment of cells with DSCG or promethazine resulted in a decreased inhibitory response to 48/80-induced release in contrast to the persistent effect of cyproheptadine under similar conditions.

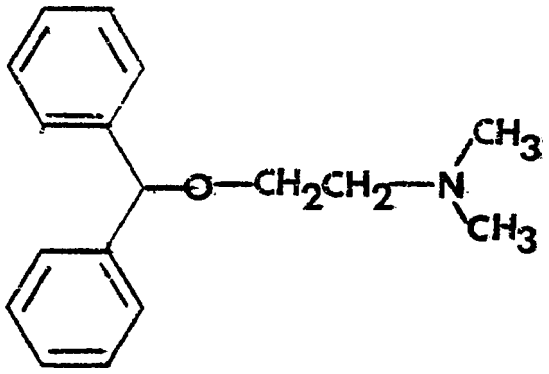
The effects of H_1-receptor stimulant, 2-pyridineethanamine, did not effect antigen-induced histamine release. The H_2-receptor stimulant, 4-methylhistamine, caused a slight depression of antigen-induced histamine release, although this inhibition was not affected by the H_2-blocking agent metiamide. Some other possible mechanisms of inhibition appear unlikely since there was no correlation between the relative potency of a series of antihistamines as antagonists of antigen-induced histamine release and their H_1-antihistaminic activity

or their local anesthetic potency.

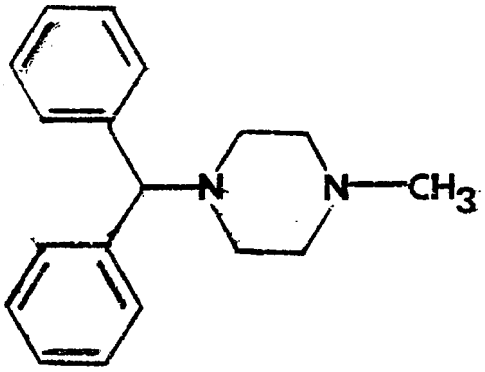
These results indicate that there are two or more sites at the rat mast cell membrane that can antagonize the calcium influx. In the current experiments, the site antagonized by the H_1-antihistamines could not be characterized in the context of any of the receptors as currently recognized by the H_1-antihistamines. Furthermore, there does not appear to be a receptor for histamine that affects histamine release on rat peritoneal cells. These findings are related to a theoretical multi-step schema for perturbation of the cell membrane which is initiated by antigen combining with cell-bound antibody that results in a calcium influx.

APPENDIX A

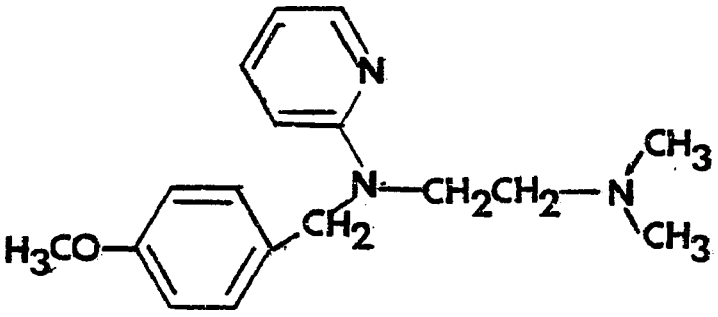
Structures



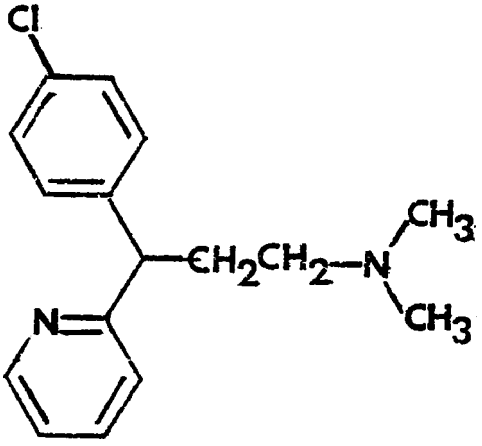
DIPHENHYDRAMINE
(ethanolamine)



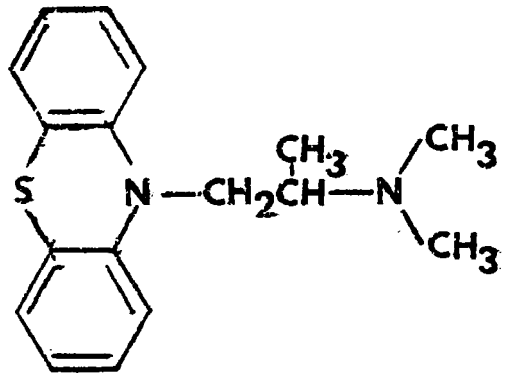
CYCLIZINE
(piperazine)



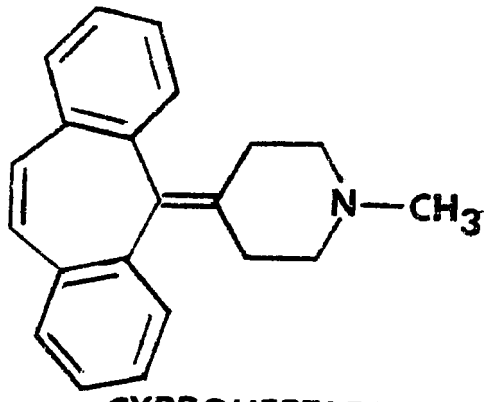
PYRILAMINE
(ethylenediamine)



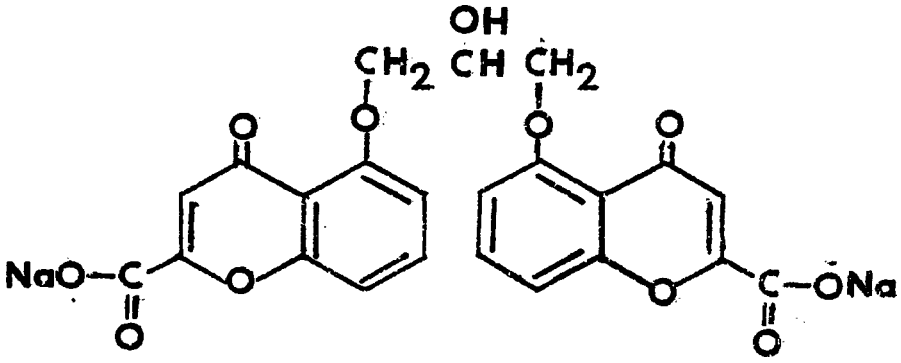
CHLORPHENIRAMINE
(alkylamine)



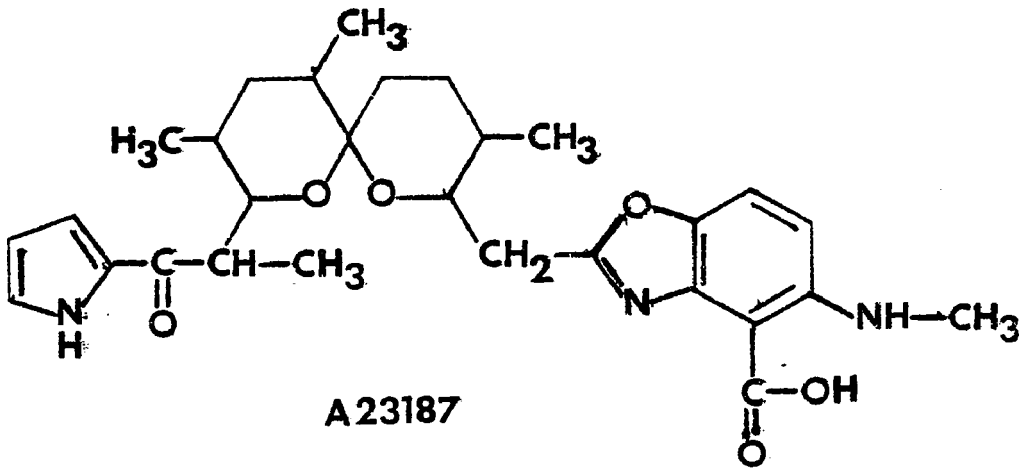
PROMETHAZINE
(phenothiazine)



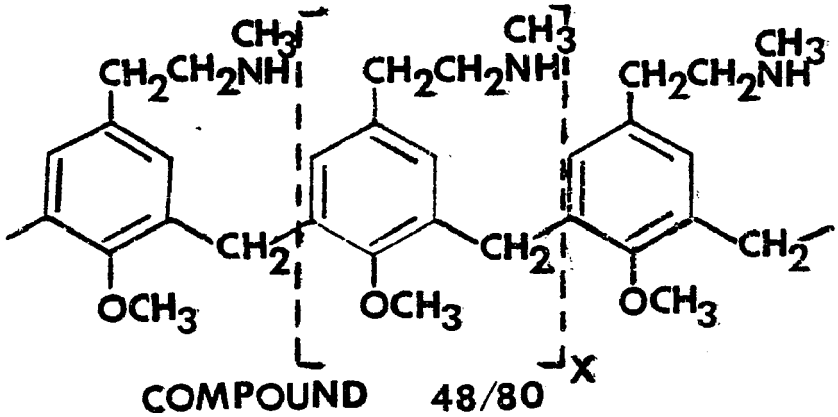
CYPROHEPTADINE



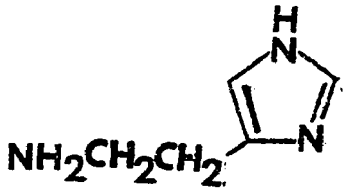
DISODIUM CHROMOGLYCATE



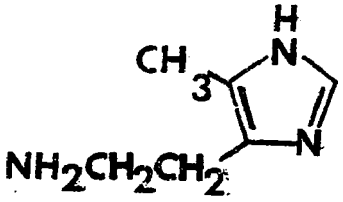
A23187



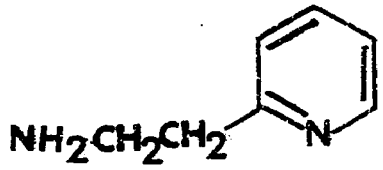
COMPOUND 48/80



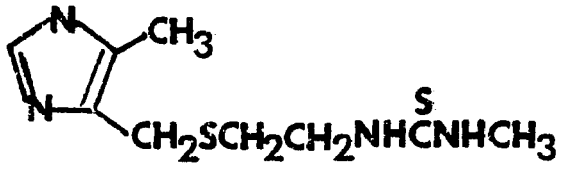
HISTAMINE



4-METHYLHISTAMINE



2-PYRIDINEETHANAMINE



METIAMIDE

APPENDIX B

FORMULATION: RPMI MEDIUM 1640

Inorganic Salts:	$Ca(NO_3)_2 \cdot 4H_2O$	100.00 mg/1
	KCl	400.00 mg/1
	$MgSO_4 \cdot 7H_2O$	100.00 mg/1
	NaCl	6000.000 mg/1
	$NaHCO_3$	2000.00 mg/1
	$Na_2HPO_4 \cdot 7H_2O$	1512.00 mg/1
Amino Acids:	L-Arginine (free base)	200.00 mg/1
	L-Asparagine	50.00 mg/1
	L-Aspartic acid	20.00 mg/1
	L-Cystine	50.00 mg/1
	L-Glutamic acid	20.00 mg/1
	L-Glutamine	300.00 mg/1
	Glycine	10.00 mg/1
	L-Histidine (free base)	15.00 mg/1
	L-Hydroxyproline	20.00 mg/1
	L-Isoleucine (Allo free)	50.00 mg/1
	L-Leucine (Methionine free)	50.00 mg/1
	L-Lysine HCl	40.00 mg/1
	L-Methionine	15.00 mg/1
	L-Phenylalanine	15.00 mg/1
	L-Proline (Hydroxy L-Proline free)	20.00 mg/1
	L-Serine	30.00 mg/1
	L-Threonine (Allo free)	20.00 mg/1
	L-Tryptophan	5.00 mg/1
	L-Tyrosine	20.00 mg/1
	L-Valine	20.00 mg/1

Vitamins:	Biotin	0.20 mg/1
	D-Ca pantothenate	0.25 mg/1
	Choline Cl	3.00 mg/1
	Folic acid	1.000 mg/1
	i-Inositol	35.000 mg/1
	Nicotinamide	1.000 mg/1
	Para-aminobenzoic acid	1.000 mg/1
	Pyridoxine HC;	1.000 mg/1
	Riboflavin	0.200 mg/1
	Thiamine HCl	1.000 mg/1
	Vitamine B_{12}	0.005 mg/1
Other Components:	Glucose	2000.00 mg/1
	Glutathione (reduced)	1.00 mg/1
	Phenol red	5.00 mg/1
	HEPES buffer*	5958.00 mg/1

* N-2-Hydroxyethylpiperazine-N^1-2 Ethane sulfonic acid

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