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CHARACTERIZATION OF HISTAMINE RECEPTORS COUPLED TO TRITIUM-CYCLIC AMP ACCUMULATION IN A VESICULAR PREPARATION OF GUINEA PIG CORTEX

City University of New York PH.D. 1985

CHARACTERIZATION OF HISTAMINE RECEPTORS COUPLED TO ^{3}H-CYCLIC AMP ACCUMULATION IN A VESICULAR PREPARATION OF GUINEA PIG CORTEX

by

MAUREEN V. NEWTON

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

CHARACTERIZATION OF HISTAMINE RECEPTORS
COUPLED TO ^{3}H-CYCLIC AMP ACCUMULATION
IN A VESICULAR PREPARATION OF GUINEA PIG CORTEX

by

MAUREEN V. NEWTON

Advisor: Lindsay B. Hough, Ph.D.

The histamine-stimulated accumulation of ^{3}H-cyclic AMP (formed by prelabeling with ^{3}H-adenine) was characterized pharmacologically in a vesicular preparation of guinea pig cortex to identify the receptors mediating this response.

Systematic variation of the preincubation time, vessel size, buffer composition, and ^{3}H-adenine labeling time significantly influenced both the basal and histamine-stimulated ^{3}H-cyclic AMP levels, and showed that individual prelabeling of aliquots in Kreb's-Ringer bicarbonate (15 mM) buffer yielded the most reproducible histamine responses.

Characterization of this histamine response showed that the H_2-antagonist cimetidine maximally blocked 80% of the response, whereas only 45% of the response could be inhibited by H_1-antagonists. A combination of H_1- and H_2-antagonists completely abolished the response. These and other findings show that both H_1- and H_2-receptors mediate

the response, but 25% of the response may require concomitant activation of both receptors.

A role for adenosine as a mediator of the histamine response was investigated. Adenosine deaminase (2.5 U/ml) decreased the basal ^{3}H-cyclic AMP levels; under these conditions the histamine response was completely abolished by cimetidine (300 uM), whereas mepyramine (3 uM) reduced the response by 30%. Thus, the H_1-response may be partially dependent upon endogenous adenosine. A combination of adenosine deaminase and the calcium chelator EGTA (2 mM) completely eliminated the H_1-component.

A "metactoid" model was developed to account for the H_2-, H_1-, and adenosine components of the histamine response. The model hypothesizes that 55% of the response is due to direct H_2-receptor stimulation, 25% is dependent on the metactoid sensitization of the H_2-response by H_1-receptors, and 20% is due to an analogous sensitization of adenosine responses by H_1-receptors. Affinity constants for both types of HA receptor antagonists, determined from fitting the above data to this model, were in agreement with literature values for these drugs.

These findings resolve previous controversies regarding the identity of the receptors mediating histamine-stimulated accumulation of cyclic AMP in brain.

Furthermore, the vesicular preparation and metactoid model developed presently may be of benefit in other studies of neurotransmitter control of cyclic AMP dynamics.

This thesis is dedicated to the two men in my life.

To my father, John E. Newton, for his contagious encouragement during my childhood education, which motivated me along a path cumulating in this manuscript. Thank you dad.

To Patrick J. Gannon, my constant companion. For his constant support and encouragement before and throughout my graduate education, I owe him my undying gratitude. As the unseen force behind this thesis, he deserves more credit than words can express. Thank you my love for all you have given me.

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1. Cyclic AMP as a Second Messenger in Brain.

1.1. Second Messenger Hypothesis.

The reception of extracellular signals and their transduction into intracellular responses is an important function of cell plasma membranes (see Rasmussen and Goodman, 1977 for review). This transduction across plasma membranes is commonly believed to occur through specific hormone-receptor interactions, and initiates elevated intracellular levels of second messengers. This may be a direct effect (e.g. conformational receptor changes initiated by hormone binding lead to ionic movement into cells) or require an intermediate transducing element, such as membrane-bound adenylate cyclase, known to mediate hormone-elevated levels of intracellular adenosine 3',5'-monophosphate (cyclic AMP). This latter mechanism is shared by many hormones and cyclic AMP is generally accepted as such an intracellular second messenger (Rasmussen and Goodman, 1977).

In the last two decades, evidence that other second messengers, particularly guanosine 3',5'-monophosphate (cyclic GMP) and calcium also operate through hormone-receptor interactions has increased (see Rasmussen and Goodman, 1977). Inositol phospholipids also act as second messengers in response to hormone-receptor stimulation. It has been suggested that inositol-1,4,5-triphosphate

(which is formed by hydrolysis of phosphatidylinositol) increases calcium mobilization and opens calcium gates (Michell, 1983). This hypothesis is the subject of intense investigation. Berridge, 1984, has suggested that both calcium-dependent and calcium-independent changes in phospholipid metabolism may occur in the same cell.

Second messengers can regulate cellular activity in two ways (Berridge, 1984). They can either activate effector systems directly, or act indirectly by modulating either the formation or mode of action of other second messengers (Berridge, 1984). Any of these intracellular messengers could influence the levels of any other one, through actions at one or more sites in the cycle of events initiated at the extracellular receptor and terminated by degradation (or removal) of the second messenger. The diverse nature of these interelationships indicates that these (and other) intracellular regulators must be considered when analysing the effects of hormones.

1.2. Adenylate Cyclase.

Membrane-bound, hormonally activated, adenylate cyclase is a complex multi-component system (see Birnbaumer and Iyengar, 1982 for review). The cyclic AMP response, generated as a result of a given hormone-receptor interaction, can be regulated at several levels of this system. Three separate components of this system

have been identified: the hormone receptor, the GTP-binding protein and the catalytic unit of adenylate cyclase. The hormone-receptor is believed to face the external side of the plasma membrane, accepting the extracellular signal. Receptors may be mobile within the membrane and appear to represent separate molecular components of this system. Another component is the GTP-binding protein. In broken cell preparations of brain and other tissues, this protein appears to function in the amplification and transmission of the hormonal signal to the third component of this system, the catalytic part of adenylate cyclase. Inhibitory hormone-receptors and GTP-binding proteins have also been reported (see Birnbaumer and Iyengar, 1982). Receptor-mediated stimulation and inhibition of adenylate cyclase requires GTP for maximal expression, although the concentration of GTP required for maximal inhibiton is higher than for activation. The GTP-binding protein and the catalytic subunit probably face the cytoplasmic side of plasma membranes.

Further complicating the possible relationships between hormone-receptor activation and cyclic AMP stimulation, recent evidence suggests that two forms of adenylate cyclase may exist in brain (see Cheung and Storm, 1982 for recent review). By sepharose affinity chromatography , bovine brain cortex has been shown to contain both calcium-sensitive and insensitive forms of

adenylate cyclase (Westcott et al., 1979). Calcium-sensitive and insensitive forms have also been identified in broken cell homogentates of guinea pig brain (Piascik et al., 1980). Brain adenylate cyclase exhibits a biphasic response to calcium, with stimulation occurring at low concentrations (< uM) and inhibition occurring at higher concentrations (> 50 uM) (Von Hungen and Roberts, 1973; Brostrom et al., 1975; Cheung et al., 1975). In broken cell membrane preparations of brain and other tissues, the common practice of including EGTA in the incubation medium presumably abolishes, or decreases, detection of hormone-receptors coupled to calcium-dependent adenylate cyclase.

1.3. Sources of Substrate ATP.

Different pools of ATP may also be present in brain tissue, which may be utilized by adenylate cyclase(s) in transducing the response to hormone-receptor interactions. These pools may be related to distinct hormone-receptor interactions. Functional compartments of adenine nucleotides, serving as precursors to cyclic AMP, have been reported in guinea pig (Chasin et al., 1973; Huang et al., 1971; Shimizu et al., 1969; Shimizu and Okayama, 1973) and mouse (Skolnick and Daly, 1975) cerebral cortex, rat ganglia (Lindl et al., 1975) and rat glial cell lines (Schultz et al., 1972). All these studies indicate that radioisotopically labeled adenine (or

adenosine) was incorporated into precursor pools of adenine nucleotides that were more highly labeled than bulk cellular ATP.

When derived from substrate pools of ATP formed through prelabeling with adenine or adenosine, the hormone-stimulated cyclic AMP could differ from the hormone-stimulated total cellular cyclic AMP response. In response to HA (100 uM) or epinephrine (10 and 100 uM), small but reproducible decreases in the specific radioactivity of ^{3}H-cyclic AMP (formed from ^{3}H-ATP prelabeled with ^{3}H-adenine) occur in guinea pig cortical slices (Chasin et al., 1973). Increases in total cyclic AMP were twice as large as those of ^{3}H-cyclic AMP when epinephrine (100 uM) was added to ^{3}H-adenine prelabeled vesicular preparations of guinea pig cerebral cortex (Chasin et al., 1974). These studies may reflect different pools of ATP coupled to hormone-receptor mediated increases in cyclic AMP.

1.4. Phosphodiesterases.

While adenylate cyclase is the only mechanism known to couple hormone-receptor interactions to the generation of cyclic AMP, differential metabolism of cyclic AMP may add yet another level of regulation of intracellular cyclic AMP levels in brain. From up to six reported multiple forms of phosphodiesterase in cerebellar brain

preparations, at least two have been reported to occur in virtually every tissue examined, including brain (see Appleman et al., 1982 and Wells and Hardman, 1977 for reviews). These two forms are commonly referred to as either the "low-K_m cyclic AMP phosphodiesterase" (K_m for cyclic AMP = 4 uM), or the "high K_m phosphodiesterase" (K_m for cyclic AMP = 100-200 uM). Obviously, the relative contribution of either of these two forms to overall cyclic AMP metabolism will depend on the cyclic AMP concentration, the relative proportion of each of these two forms present and any possible effects of one enzyme on the other (e.g. maximum stimulation of the low K_m form masks the properties of the high K_m form). These complicating factors have not been commonly considered in analysing the properties of phosphodiesterase in brain, or in other tissues. The composition and properties of phosphodiesterases vary between tissues and cell types (see Appleman et al., 1982).

In the absence of phosphodiesterase inhibitors, both cyclic GMP and calcium may act on the high K_m phosphodiesterase in brain and alter the apparent cyclic AMP levels seen in response to hormonal stimulation. In most mammalian tissues, the high K_m enzyme has a greater V_{max} for cyclic AMP than for cyclic GMP, but a lower apparent K_m for cyclic GMP than cyclic AMP (3-8 uM vs 200 uM respectively) (Appleman et al., 1982). Hydrolysis of

cyclic AMP is competitively inhibited by cyclic GMP and vice versa, with K_i values similar to their respective Km values (Wells and Hardman, 1977). These observations suggest that a single catalytic site is involved in the hydrolysis of both substrates. This phosphodiesterase activity is also associated with the calcium-dependent activator protein calmodulin (Appleman et al., 1982). Calmodulin requires calcium and magnesium for activity, and neither calmodulin without calcium, nor calcium without calmodulin, can stimulate cyclic AMP metabolism (Wells and Hardman, 1977). Since the high K_m form has been reported to represent over 90% of phosphodiesterase activity in rat brain cortex (Kakiuchi et al., 1975), and appears to be abundant in postsynaptic densities of rat neocortex (Ariano and Appleman, 1979), changes in cyclic GMP and calcium should be considered when interpreting the mechanisms of hormone-receptor stimulated cyclic AMP levels.

The importance of the low K_m phosphodiesterase in the metabolism of cyclic AMP is unclear. This enzyme does not appear to metabolize cyclic GMP, or be activated by calmodulin. It displays negative cooperativity and its activity may change in response to some hormones and may increase in response to elevated substrate levels in intact cells (see Wells and Hardman 1977 for review). These two latter phenomena do not appear to have been

observed for brain and their functional significance is unknown.

Theoretical simulations have revealed that phosphodiesterase(s) may have a great potential in regulating the magnitude and kinetic characteristics of hormone-receptor mediated changes in cyclic AMP levels (Erneux et al., 1980; Reynolds, 1982). However, the physiological relevance of this potential regulatory control mechanism remains unclear.

Experiments with high concentrations of different phosphodiesterase inhibitors support the suggestion that hormonally-stimulated cyclic AMP synthesis can occur through coupling to different ATP pools in brain (Mah and Daly, 1976; Schultz and Daly, 1973b). In guinea pig cortical slices, papaverine and 4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidinone (RO-20-1724) (0.2 mM) potentiated the adenine prelabeled ^{14}C-cyclic AMP response to HA (0.1 mM), while only RO-20-1724 was effective in potentiating the response to adenosine (Mah and Daly, 1976). HA-stimulated (0.1 mM) total cellular and prelabeled ^{3}H-cyclic AMP are also differentially affected by phosphodiesterase inhibitors (Schultz and Daly, 1973b). In this study, while papaverine (0.5 mM) caused a two fold rise in both HA-stimulated total and ^{3}H-cyclic AMP levels, isobutylmethylxanthine (IBMX, 1 mM) potentiated HA-elicited accumulations of total cyclic AMP to a greater

extent (4 fold) than the accumulation of radioactive cyclic AMP (2 fold). The potentiating effects of papaverine and IBMX on accumulations of total cyclic AMP were not additive. Whether or not the possible differences in cyclic AMP responses described are real, or merely an artifact caused through use of single saturating concentrations of agonists and phosphodiesterase inhibitors, remains to be demonstrated.

In summary, the overall response to hormonally-regulated cyclic AMP levels is determined by the relative rates of cyclic AMP metabolism and synthesis, which, as discussed above, are subject to complex regulatory control. While phosphodiesterases are the only known pathway for cyclic nucleotide degradation, little is known about how these enzymes regulate cyclic nucleotide levels or how their own activity is regulated in intact tissues. The relationship of possibly different adenylate cyclase effector mechanisms to hormonally-mediated increases in cyclic AMP synthesis, and the subsequent degradation of this second messenger, remains to be demonstrated.

1.5. Physiological Role of Cyclic AMP in the CNS.

It is commonly believed that activation of cyclic AMP-dependent protein kinase and subsequent protein phosphorylation are the biochemical mechanisms mediating cyclic AMP action in all tissues. Although these

processes are presumably involved in the physiological control of CNS functions through hormone-receptor interactions, little direct evidence for this conclusion is available (Dunwiddie and Hoffer, 1982).

Cyclic AMP is probably a presynaptic modulator in brain. Cyclic AMP appears to be involved in the short-term activation of tyrosine hydroxylase (the rate-limiting enzyme in the synthesis of catecholamines). Stimulation of peripheral and central catecholamine pathways result in increased tyrosine hydroxylase activity which is mimicked by treatments that increase cyclic AMP levels (see Dunwiddie and Hoffer, 1982). Recently, it has been suggested that a calcium/calmodulin-dependent protein kinase, in the presence of an activator protein, can also increase tyrosine hydroxylase activity (see Forn, 1984).

Another presynaptic substrate for cyclic AMP-dependent protein kinase, synapsin 1 (previously called Protein 1), has been extensively characterized and is localized exclusively to the synaptic region. Depolarizing conditions stimulate phosphorylation of synapsin 1, through a mechanism dependent on extracellular calcium (Kruger et al., 1977). Many hormones, including HA and adenosine, have no apparent effect on phosphorylation of synapsin (Forn and Greengard, 1978). It has been suggested that this protein can be independently phosphorylated through calcium and cyclic AMP-dependent mechanisms (Forn

1984). The physiological function of this protein is currently unknown (see Forn, 1984).

Cyclic AMP has also been suggested to play a role in the presynaptic release of neurotransmitters. Many transmitters appear to regulate their own (or other transmitter) release through an action at a presynaptic receptor site(s) (autoreceptors). Many of these agents stimulate changes in cyclic AMP levels. Whether or not these changes are related to control of presynaptic release processes is the subject of intense controversy (see Dunwiddie and Hoffer, 1982).

A postsynaptic action of cyclic AMP is commonly thought to account for many hormone-receptor mediated changes in cyclic AMP levels. In most tissues, including brain, cyclic AMP predominantly hyperpolarizes or inhibits firing of most cell types studied (see Siggins, 1982 for review). While contradictory evidence exists, much information suggests that noradrenaline acts through -receptors to increase cyclic AMP levels in the cerebellum which then depress cerebellar Purkinje cell spontaneous discharge (see Siggins, 1982 and Dunwiddie and Hoffer, 1982). Noradrenaline may also inhibit the firing of hippocampal pyramidal cells through a similar mechanism (Siggins, 1982). H_2-mediated activation of adenylate cyclase may also initiate electrophysiological changes in the hippocampus (see Introduction 6.3.1.).

Cyclic AMP also appears to be involved in the control of microtubule function in brain. A cyclic AMP-dependent protein kinase catalyses the phosphorylation of MAP 2, a microtubule-associated protein (Sloboda et al., 1975). Phosphorylation of this protein appears to inhibit the rate and extent of microtubule assembly (Jameson et al., 198Ø). Thus cyclic AMP may have a role in regulating cytoskeletal function.

Another possible role of cyclic AMP is as a regulator of cellular metabolism. Cyclic AMP is probably involved in receptor-mediated mobilization of cerebral glycogen stores, through phosphorylation of phosphorylase a to b (Dunwiddie and Hoffer, 1982). This effect occurs in neurons but may be predominantly localized to glial cells (Dunwiddie and Hoffer, 1982).

In conclusion, cyclic AMP appears to regulate several physiological responses in brain. Although several of these responses may be involved in neurotransmission, others, such as metabolic effects, may be related to nonneuronal function.

2. Adenosine in Brain.

In addition to their established role in metabolic regulation, adenine derivatives have been postulated to have a role in neurotransmission (e.g. McIlwain, 1972; Stone, 1981). The presence in brain of specific uptake mechanisms for adenosine may support a neurotransmitter role for this nucleoside (see Stone, 1981). Adenine, or adenosine, are rapidly taken up into brain tissue by a high affinity carrier-mediated mechanism (K_m around 3 uM), a lower affinity system (K_m around 250 uM) and, above 10 uM, partly by a non-saturable mechanism. These uptake processes, or some components thereof, can be blocked by dipyridamole, papaverine and other agents, but not by methylxanthines.

Once inside cells, adenine and adenosine are rapidly converted to nucleotides, mainly ATP and AMP. As discussed (see Introduction 1.3) extracellular adenine and adenosine may be incorporated into a pool of ATP that does not readily equilibrate with other intracellular pools. Endogenous pools of free intracellular adenosine have not been demonstrated in brain (see Stone, 1981). Indeed, even in the presence of high extracellular concentrations of adenosine, intracellular concentrations are kept to a minimum (Stone, 1981).

Adenosine can be released from brain, but these release mechanisms have properties different than the mechanism conventionally associated with transmitter release, where potassium depolarization readily and promptly evokes a calcium-dependent release mechanism (see Stone, 1981 for review). While electrically-evoked adenosine release is dependent on external calcium, potassium-evoked release is not (Stone, 1981). The release produced by ouabain may involve an influx of both sodium and calcium, and does not appear to involve this agents ability to inhibit membrane ATPase activity (Stone, 1981). Efflux of adenosine from brain slices is also markedly increased by electrical stimulation, depolarizing agents, glutamate and conditions of metabolic stress, including low O_2, glucose or calcium (McIlwain, 1972). All of these stimlui increase cyclic AMP levels in brain. It should be noted, however, that all the findings described above were based on studies measuring changes in radioisotopically prelabeled nucleotide pools. Whether or not these manipulations also changed endogenous purine distribution has not been demonstrated. The functional significance of labeled-adenosine efflux from brain is unknown, but it may have a role in protecting cerebral tissues during hypoxia or anoxia (Phillis and Wu, 1983).

It has been suggested (Stone, 1981) that stimuli releasing adenosine may act, at least in part, through

stimulating an increase in intracellular cyclic AMP. On hydrolysis of this compound, adenosine is produced (via ADP), which can then exert a further action at an extracellular receptor. ATP, released with neuro-transmitters on membrane depolarization, might also serve as a source of extracellular adenosine. Interestingly, however, depolarization-induced cyclic AMP accumulation may be due to adenosine release which is not derived from hydolysis of released ATP (Pons et al., 1980).

Two adenosine receptors, R and P, have been identified by their influence on adenylate cyclase activity (see Stone, 1981 and Londos et al., 1983 for recent reviews). High concentrations of adenosine (> 100 uM) and ribose-modified analogues, such as 2-deoxyadenosine, inhibit cyclic AMP synthesis through an unknown methylxanthine-resistant mechanism. This action, designated as occurring through a P-site, requires an intact nucleoside structure and only adenine can serve as the purine moiety.

R-receptors have been identified by their influence on adenylate cyclase activity (Wolff et al., 1981) and binding studies (Daly, 1983). To interact with these receptors, agonists require a nucleoside structure, with an intact ribose ring and show an absolute dependence on nitrogen at C6, in the purine moiety (Wolff et al., 1981). In broken cell membrane preparations of brain and other tissues, adenosine stimulation of R-receptors results in

either activation (EC_{50} = 0.5 - 25 uM) or inhibition (EC_{50} = 25 - 100 nM) of adenylate cyclase activity. Both these adenosine responses require GTP, and are antagonized by methylxanthines (e.g. theophylline and IBMX). Activation or inhibition of adenylate cyclase activity may occur through different subtypes of R-receptors, since the rank order of potency of R-agonists appears to be different between these two effects (Londos et al., 1983), and these two sites may also be distinguished by binding studies (Daly, 1983).

Adenosine stimulates cyclic AMP accumulation in slices of many different regions of brain (Daly, 1979). Adenosine also stimulates cyclic AMP accumulation in glial cell cultures (Van Calker et al., 1979; Schultz et al., 1972) and neuroblastoma cell lines (Blume and Foster, 1975; Green and Stanberry, 1977; Perit et al., 1976). The physiological roles of adenosine-mediated cyclic AMP accumulation are unknown.

Adenosine-stimulated cyclic AMP accumulation in brain slices appears to be mediated through activation of an extracellular receptor (see Stone, 1981). Adenosine-stimulated cyclic AMP accumulation can be potentiated by adenosine uptake inhibitors (Daly, 1976; Huang and Daly, 1974) and can be mimicked by adenosine receptor agonists (Daly, 1976; Mah and Daly, 1976). The observation that theophylline and other methylxanthines block adenosine-

mediated cyclic AMP accumulation in brain slices, first noted by Sattin and Rall (1970), supports the hypothesis that adenosine increases cyclic AMP through activation of an extracellular receptor.

In brain slices, combinations of adenosine with either norepinephrine or HA commonly cause greater than additive effects on cyclic AMP accumulation (see Daly, 1977 for review). These synergistic interactions do not appear to occur in different types of cell cultures (see Dunwiddie and Hoffer, 1982 for refs.). In broken cell homogenates of rat striatum, activation of adenylate cyclase by adenosine and dopamine is additive (Premont et al., 1979). It has therefore been suggested that the synergistic interactions of these agents on cyclic AMP accumulation requires different cell types and may involve the release of other neurohumoral substances (Dunwiddie and Hoffer, 1982).

Interestingly, although adenosine increases cyclic AMP accumulation in guinea pig, rat and rabbit cortical brain slices, adenosine does not stimulate adenylate cyclase in broken cell preparations of rat cortex (Premont et al., 1979). Indeed adenosine inhibits adenylate cyclase activity in broken cell preparations of rat cortex (Cooper et al., 1980). It is possible that both inhibitory and stimulatory adenosine receptors are coupled to the regulation of cyclic AMP accumulation in brain slices, but

an inhibitory site has not been observed.

Adenosine has electrophysiological effects on brain, which may support a role for this nucleoside in neurotransmission. Iontophoretically applied adenosine is a potent depressant of spontaneous neuronal activity in most brain regions studied (see Phillis and Wu, 1983). It has been suggested that the electrophysiological effects of adenosine may reflect adenosine inhibition of neurotransmitter release, via an action on a presynaptic adenosine receptors (see Phillis and Wu, 1983). The ability of adenosine to inhibit neurotransmitter release may result from a reduction in the calcium permeability of the nerve terminal plasma membrane (Phillis and Wu, 1983). The evidence that these electrophysiological effects are mediated by increased cyclic AMP accumulation is weak (Dunwiddie and Hoffer, 1982).

3. Histamine as a Neurotransmitter in Brain.

Histamine is generally accepted as a neurotransmitter in brain (for recent review see Hough and Green, 1984). Several criteria for a neurotransmitter function of HA have been met. The brain contains specific enzymes for the synthesis (histidine decarboxylase) and metabolism (HA methyltransferase) of HA. Histamine has a nonuniform regional distribution and appears to turn over rapidly at rates that vary among brain regions (Hough et al., 1984). Like other biogenic amines, HA is released by potassium ions in a calcium-dependent process. Neurons respond to iontophoretically applied HA. In the cerebral cortex and hippocampus, HA usually causes a depression of firing, while in the hypothalamus many neurons are excited by HA (see Hough and Green, 1984).

Early studies with electrolytic lesions and biochemical studies suggested that HA containing neurons project to many telecephalic regions (see Garbarg et al., 1980). With the recent development of histochemical methods for specific vizualiation of histaminergic neurons, these studies have been largely confirmed. Posterior hypothalamic areas probably contain the majority of HA-containing cell bodies in rat brain. Immunohistological techniques, with an anti-histidine decarboxylase antibody, suggest that the tuberal magnocellular, caudal magnocellular, posterior and

dorsomedial hypothalmic nuclei of the hypothalamus contain HA cell bodies (Watanabe et al., 1984). By use of a HA - antibody, HA cell bodies were also observed in the ventral tegmentum and regions lateral and ventral to the posterior hypothalamic nuclei (Steinbusch and Mulder, 1985; Dirks et al., 1984). Lesioning studies suggested the presence of HA containing cell bodies in these areas as well as in the rostral mesencephalon (Pollard et al., 1978), but these were not noted using immunocytochemistry of histidine decarboxylase (Watanabe et al., 1984). HA cell bodies have also been shown in the ventral horn of the spinal cord (Leslie et al, 1984).

It seems likely that HA is also present in a nonneuronal compartment of brain. Lesioning studies can result in total depletion of histidine decarboxylase, yet only partial reduction of HA content distal to the lesion (Schwartz et al., 1980). Subcellular distribution studies indicate that a large fraction of brain HA is contained in nerve endings which also contain most of the brain histidine decarboxylase activity (see Hough and Green, 1984). This would suggest that lesion-induced neuronal changes are more accurately reflected in measurements of histidine decarboxylase activity.

The possibility that brain mast cells, with properties similar to peripheral mast cells, serve as the only nonneuronal storage site for brain HA appears unlikely.

Recent observations indicate that mast cells are only present in high numbers in thalamic areas of rat brain (Goldschmidt et al., 1985). This observation does not exclude the possibility that HA may be stored in a 'mast cell-like' cell such as the neurolipomastocytoid cell (Ibrahim et al., 1979). If such cells have a high HA content, they could contribute to overall HA levels in some brain areas. The function of mast cells and putatively similar cell lines in brain is unknown.

Other cell types, including glial and vascular elements, may also store HA but these possibilities have not been extensively addressed. Blood vessels contain HA and microvasculature also contains small amounts of histidine decarboxylase and histamine-methyltransferase activity (Karnushina et al., 1979 and 1980; Robinson-White and Beaven, 1982). The contribution of HA stored in vascular elements to brain HA levels is unknown.

Histamine has effects on several biochemical responses in brain (see Schwartz et al., 1982 and Hough and Green, 1984 for recent reviews). As in peripheral systems, most of these responses have been attributed to HA-stimulation of either of two specific HA-receptors, designated H_1- and H_2- (Black et al., 1972). Stimulation of several biochemical responses, including glycogenolysis, phosphoinositol turnover, cyclic GMP, cyclic AMP and calcium mobilization have been attributed, or suggested,

to involve H_1-receptor stimulation (see Introduction 5. and 6.3.). Conversely, the only well characterized biochemical response coupled to H_2-receptor stimulation is activation of adenylate cyclase (see Introduction 6.2.).

A third HA-receptor subtype (H_3) has recently been postulated to control HA release from presynaptic sites in rat cerebral cortical slices (Arrang et al., 1983). HA inhibited potassium-induced release of labeled HA formed from incubation with labeled histidine, and this effect could not be ascribed to either H_1- or H_2-receptors (Arrang et al., 1983). In particular, this HA response occurred at concentrations of HA (EC_{50} = 40 nM) considerably lower than its EC_{50} values at H_2- (uM) or H_1-receptors (0.1-10 uM) and was blocked by burimamide (an H_2-antagonist) and impromidine (an H_2-agonist). This postulated site of potential HA-action is attractive since it would provide evidence for HA-autoreceptor feedback inhibition, as has been described in the presynaptic regulation of other neurotransmitter systems.

Several physiological and behavioral functions of brain HA have been suggested. HA has been implicated in the control of cerebral circulation (Gross, 1981 and 1982), drinking and feeding behavior (Leibowitz 1979), cardiovascular regulation, thermal regulation, self-stimulation, arousal, and neuroendocrine regulation (see Hough and Green, 1984 and Roberts and Calcutt, 1983).

Some of these responses are considered below.

Histamine may function as a mediator of arousal (see Hough and Green, 1984; Roberts and Calcutt, 1983). Consistent with this view, microinfusion of HA into the superchiasmatic nucleus caused rats to become more active, while systemic administration of the histidine decarboxylase inhibitor α-fluoromethylhistidine (100 mg/Kg) caused a decrease in arousal time associated with an increase of slow wave sleep (Wada et al., 1984). The sedative effects of H_1-antagonists might therefore reflect inhibition of H_1-receptors in brain. However, although H_1-antagonists have a high affinity at H_1-receptors (see Introduction 3.2), at higher concentrations these drugs act as local anesthetics and act at many other sites including blockade of muscarinic- (see Van Den Brink and Lein, 1977) and H_2-receptors (see Introduction 3.3.2). In addition, a simple relationship between peripheral H_1-receptor blockade and sedation has not been observed in man (Carruthers et al., 1978; Peck et al., 1975).

H_2-receptor activation could be involved in the maintenance of normal mentation (Hough and Green, 1984). Although minor in incidence, the H_2-antagonists, cimetidine and ranitidine (which are widely used in the treatment of ulcers) caused dose-related mental symptoms including confusion, auditory and visual hallucinations (see Epstein, 1984; Hughes et al 1983; Mani et al., 1984;

Schentag et al., 1979; Silverstone, 1984). Recent evidence disputes the original contention that cimetidine does not pass the blood brain barrier (Colboc et al., 1982) and CNS levels of cimetidine could significantly occupy H_2-receptors (see Hough and Green, 1984).

Endogenous HA may function in thermoregulation (see Lomax and Green, 1981; Roberts and Calcutt, 1983). In general, intracerebroventricular (icv) HA caused a dose-dependent decrease in body temperature in several mammalian species. Both H_1- and H_2-receptors appear to be involved in this response (see Roberts and Calcutt, 1983). In contrast, icv administration of HA agonists caused hyperthermia in chloral-anaethetized rats, an effect probably mediated by H_2-receptors (Colboc et al., 1982). Intrahypothalmic injections of HA caused a decrease in body temperature in conscious rats, an effect blocked by both H_1- and H_2-antagonists (Bugajski and Zacny, 1981). It has been suggested that HA induces hypothermia through H_1-receptor stimulation by lowering the set point of the hypothalamic thermostat and that H_2-receptors are involved in a heat loss mechanism (Bugajski and Zacny, 1981).

HA may regulate the release of several pituiary hormones, including gonadotropins and antidiuretic hormone (see Hough and Green, 1984; Roberts and Calcutt, 1983). Histamine is involved in the control of body fluid balance. Both H_1- and H_2-receptors may be involved in

HA-increased drinking behavior (Leibowitz, 1979). Microinjections of HA near the superoptic nucleus decreased urine volume, an effect blocked by the H_1-antagonist mepyramine (Bennett and Pert, 1974). This decrease in urine output is probably due to HA-stimulated release of antidiuretic hormone from the posterior pituitary (Bhargava et al., 1973; Eriksson and Tuomisto, 1978; Tuomisto et al., 1980). This response is antagonized by mepyramine (icv) (Bhargava et al., 1973), also imputing the involvement of H_1-receptors in the control of body fluid balance.

Both H_1- and H_2-receptors may be involved in HA-induced prolactin release. HA implants in the rostral and mediobasal hypothalamus induced prolactin release in conscious male rats (Alvarey and Donoso, 1981). Intracerebroventricular HA, cimetidine or metiamide increased plasma prolactin levels in rats (see Roberts and Calcutt, 1983). This stimulatory effect of H_2-antagonists was not correlated with the ability of these compounds to act as H_2-receptor antagonists (Netti et al., 1983), and may involve an interaction with serotonin receptors (Beck and Libertun, 1983). However, it seems possible that H_2-receptors mediate increased prolactin release in response to HA, in male rats. The stimulatory effect of HA was blocked by concomitant infusion of cimetidine at concentrations that have no effect on basal prolactin

release (Matzen et al., 1984) and was mimicked by other H_2-receptor agonists (see Roberts and Calcutt, 1983). In contrast, icv administration of 4-methyl-HA or H_1-antagonists inhibited suckling-induced prolactin release in female rats, an effect not blocked by metiamide (Arakelian and Libertun, 1977). Similarly, systemic administration of mepyramine blocked prolactin release in response to intravenous HA infusion in man, while cimetidine potentiated this HA response (Knigge et al., 1982 and 1984). It is unclear whether these effect of H_1-antagonists occur through H_1-receptor blockade or through other actions of these compounds. Thus, while both H_1- and H_2-receptors may control HA-induced prolactin release, it seems likely that the effects of HA may be species dependent and vary under different steroid states (Roberts and Calcutt, 1983).

In summary, a large part of HA actions in brain are probably attributable to its function as a neuro-transmitter. However, HA may also mediate non-neuronal responses. As with other biogenic amines, addressing the cellular locus and mechanism of action of HA-mediated effects in brain awaits development of suitable preparative techniques.

4. Histamine Receptors in Brain Characterized by Binding Studies.

In both brain and peripheral tissues, two classes of HA receptors, H_1- and H_2-, appear to mediate the various biological responses to HA (Green and Hough, 1980). While the presence of H_1-receptors has been demonstrated in brain and peripheral tissues using radiolabeled binding techniques, similar attempts at identifying H_2-receptor binding sites have proved unsuccessful (see below). H_1-binding studies can be correlated with functional response and, as such, may aid in determining the receptors underlying the HA-induced responses in the CNS. For example, the affinity constants of H_1-antagonists in inhibiting ^{3}H-mepyramine binding in guinea pig smooth muscle are similar to those inhibiting contractile responses to HA (see Table 1 and Hill and Young, 1977).

4.1. H_1-Receptor Binding.

4.1.1. Pharmacological Characterization of H_1-Binding Sites.

Both high (nM) and low (uM) affinity H_1-antagonist binding sites for labeled mepyramine or doxepin have been described and/or inferred after studies of both rat and guinea pig brains (Hadfield et al., 1983; Hill and Young, 1980; Taylor and Richelson, 1982; Tran et al., 1981). The high affinity binding site appears to identify H_1-

receptors. It is unclear whether the low affinity site represents a distinct receptor population. Obviously, the relative contribution of these sites to total ^{3}H-mepyramine binding will depend on the relative proportion of each of these two forms present, the labeling concentration used, and any possible effects of one site on the other (eg. negative cooperativity). In most studies, these complicating factors have not been considered in analysing the properties of H_1-receptor binding sites.

Another variable influencing the apparent dissociation constant of H_1-antagonists is the concentration of membranes used in the assays. In rat brain, using ^{3}H-mepyramine, Taylor and Richelson (1980) have demonstrated that the apparent dissociation constants of doxepin, amitryptyline and nortriptyline were decreased by an order of magnitude when protein concentration was lowered from 2.0 to 0.7 mg/ml. Similarly the IC_{50} for astemizole in displacing ^{3}H-mepyramine (4 nM) binding from guinea pig cerebellum changed from 40 to 4 nM when the incubation volume was increased from 1 to 10 ml (Laduron et al., 1982). These decreases in apparent antagonist dissociation constants with protein dilution imply that at high protein concentrations a significant amount of antagonist was bound to protein such that the free drug concentration was less than the concentration added. It

seems likely that reported K_b values for doxepin and amitryptyline (Table 1) in guinea pig brain are overestimated, particularly since the K_b values for these agents in inhibiting H_1-induced contraction of the guinea pig ileum were 10 fold lower (Table 1).

Given the differences outlined above, there is generally good agreement between the apparent affinity constants of ligands determined by displacing selectively low concentrations of ^{3}H-mepyramine from membrane preparations of guinea pig brain and small intestine (see Table 1 and Hill and Young, 1977). The IC_{50} values of H_1-antagonists determined from competition with ^{3}H-doxepin (0.5 nM) binding were similar to those determined from displacement of ^{3}H-mepyramine (4 nM) binding in guinea pig brain membranes (Tran et al., 1981). H_1-antagonist displacement studies also appear to show that ^{3}H-doxepin (0.08 nM) labels sites with similar characteristics to those labeled by ^{3}H-mepyramine, although minor differences were noted (Taylor and Richelson, 1982). All membranes studied discriminate between the stereoisomers of chlorpheniramine (see Table 1 and Taylor and Richelson, 1982).

Species differences occur in both the affinity, regional distribution and B_{max} of antagonist binding (Chang et al., 1979a). B_{max} values for ^{3}H-mepyramine binding also vary between guinea pig brains (Hill and

Table 1. Comparison of the apparent dissociation constants of several H_1-antagonists in guinea pig brain and intestinal smooth muscle determined from inhibition of ^{3}H-mepyramine binding and from inhibition of H_1-receptor mediated responses.

Tissue	Apparent dissociation constants (nM)				
	Brain			Ileum	
Region	cortex	cortex **	hippo-campus ***		
Method	binding*	cyclic AMP accumulation		binding*	contr-action
Antagonist					
Mepyramine	0.5 a	1.8 b	2.0 c	1.4 d	0.4 f
d-Chlorpheniramine	1.4 a	2.3 b	-	2.4 d	1.3 b
l-Chlorpheniramine	130 a	490 b	-	190 d	560 b
Promethazine	3.2 a	1.7 b	25 c 22 h	44 e	1.2 f
Diphenhydramine	13.7 a	-	50 c	-	7.2 f
Doxepin	0.5 a	-	-	-	0.06 g
Amitriptyline	1.6 a	-	-	-	0.08 g

Shown is a comparison of the apparent dissociation constants of selected H_1-antagonists in inhibiting ^{3}H-mepyramine binding or H_1-mediated cyclic AMP accumulation in guinea pig brain.

* Apparent dissociation constants were commonly calculated from competition with a single concentration of ^{3}H-mepyramine using the relationship:

$$K_b = \frac{IC_{50}}{(1+ [A]/K_a)}$$

where:

K_b = apparent antagonist dissociation constant.

IC_{50} = concentration of inhibitor causing 50% reduction in specific ^{3}H-mepyramine binding.

A = ^{3}H-mepyramine concentration.

K_a = dissociation constant of mepyramine.

** Determined from the inhibition of HA-potentiation of the cyclic AMP response to exogenous adenosine.

*** IC_{50} values were determined from the concentration of H_1-antagonists required to inhibit 50% of that portion of the cyclic AMP response to HA sensitive to inhibition by these agents. Dissociation constants were calculated assuming an analagous relationship to that shown above for binding studies (*).

Data taken from ref no.:

a. Chang et al., 1979a; b. Hill et al., 1981a; c. Palacios et al., 1978a; d. Chang et al., 1979b; e. Hill and Young, 1977; f. Triggle and Triggle, 1976; g. Figge et al., 1979; h. Trung Tuong et al. 1980.

Young, 1978). Displacement of ^{3}H-mepyramine (1-2 nM) binding by H_1-antagonists revealed that guinea pig membranes show higher affinity (up to 10 fold) than comparable rat preparations for d-chlorpheniramine, triprolidine and promazine (Chang et al., 1979a; Hill and Young, 1980). d-Chlorpheniramine and triprolidine were also more potent in displacing ^{3}H-doxepin (0.5 nM) binding from guinea pig compared to rat membranes preparations of brain (Toll and Snyder, 1982). These latter differences were maintained when H_1-binding sites were solubilized (Toll and Snyder, 1982).

The proportion of promethazine-sensitive ^{3}H-mepyramine binding sites was higher when 6,000 g membrane preparations were utilized and bound material collected by centrifugation (Hill et al., 1978) compared to triprolidine-insensitive binding sites utilizing 50,000 g membranes and filtration (Chang et al., 1979b). It is unknown whether this difference reflects differences in the membrane fraction, or different properties of the masking ligand. For three H_1-antagonists, mepyramine, methapyrilene and triprolidine, Hill coefficients determined from inhibition of ^{3}H-mepyramine binding were significantly less than one (Chang et al., 1979a; Hill and Young, 1980). These findings may be related to the sodium phosphate buffer used in these studies (see below) and/or to the heterogeneity of ^{3}H-mepyramine binding sites.

Previous studies demonstrating weak effects of HA at ^{3}H-mepyramine binding sites, compared to its ability to stimulate H_1-mediated responses, may be related to the use of sodium containing buffers (Chang and Snyder, 1980). In the guinea pig brain, sodium decreases HA (5 uM) inhibition of mepyramine (1 nM) binding, in a concentration-dependent fashion (Chang and Snyder, 1980). In the presence of 100 mM NaCl, the affinity of H_1-agonists for mepyramine binding sites is decreased by approximately 10 fold (Chang and Snyder, 1980). In the same study, GTP also decreased the potency of HA and 2-aminoethylpyridine (a selective H_1- agonist) in reducing ^{3}H-mepyramine binding. The divalent cations manganese and magnesium, but not calcium, increased the potencies of HA and 2-aminoethylpyridine in reducing ^{3}H-mepyramine binding. None of these manipulations changed ^{3}H-mepyramine binding characteristics.

Agonist-specific regulation of hormone and neurotransmitter receptor binding by guanine nucleotides often appears to reflect a coupling of the receptor to an adenylate cyclase system (see Chang and Snyder, 1980). However, guanine nucleotides also influence receptor binding interactions for which no link to adenylate cyclase has been demonstrated. For example, the cerebellum of guinea pigs contains the highest levels of H_1-antagonist binding (Hill et al., 1978; Tran et al.,

1978), which does not appear to be associated with large HA-mediated increases in cyclic AMP (Ohga and Daly, 1977). The possibility that species differences in H_1-antagonist binding also reflect the degree of HA-stimulated cyclic AMP accumulation should also be considered in light of the regional differences in binding and cyclic AMP stimulation seen within the same species.

As noted above, use of higher labeling ligand concentrations have revealed a second, lower affinity binding site for H_1-antagonists in membrane preparations from both rats and guinea pigs (Hadfield et al., 1983; Hill and Young, 1980; Taylor and Richelson, 1982; Tran et al., 1981). With high concentrations of ^{3}H-doxepin (4 nM) and 0.2 uM triprolidine to mask high affinity H_1-binding sites, IC_{50} values for most H_1-antagonists in displacing remaining ^{3}H-doxepin binding in guinea pig brain membranes were in the uM range and no stereo-selectivity between stereoisomers of chlorpheniramine was noted (Tran et al., 1981). This low affinity site does not correspond to any known binding site or functional HA response (Tran et al., 1981). In guinea pig cortex, extrapolation of a Scatchard plot of ^{3}H-doxepin binding suggested that the B_{max} of the low affinity (25.8 nM) was approximately 10 times the high affinity (0.26 nM) binding site (87 vs 6.7 pmol/g wet weight; Tran et al., 1981).

4.1.2. Distribution and Localization.

In all species and tissues studied, brain has the highest density of H_1-receptors, as assessed by the number of ^{3}H-mepyramine binding sites. (Chang et al., 1979a and b). In the guinea pig brain, maximum binding is found in the cerebellum, with lower levels in the hypothalamus, cortex and hippocampus and small amounts in the caudate nucleus, brain stem and spinal cord (Hill et al., 1978; Tran et al., 1978). In contrast, the rat has lowest levels of binding in the cerebellum and highest in the hypothalamus (Chang et al., 1979). These studies demonstrate the lack of correlation between presumed histaminergic nerve terminals and H_1-receptors. In all species studied, the highest levels of HA are found in the hypothalamus with lowest levels in the cerebellum (see Hough and Green, 1984 for ref).

H_1-receptors have been visualized in the molecular layers of the cerebellum and the dentate gyrus of the hippocampus by autoradiography with ^{3}H-mepyramine, where, as in membrane preparations, the number of binding sites appeared lower in the rat than in the guinea pig (Palacios et al., 1979 and 1981a). From the localization of receptor densities, the authors concluded that H_1-receptors were preferentially associated with neuronal systems, rather than glial, mast cells or blood vessels (Palacios et al., 1979). However, intrahippocampal kainic

acid injections, medial forebrain bundle lesions, and fimbria and fornix transections did not significantly decrease ^{3}H-mepyramine binding in the rat hippocampus (Chang et al., 1980). These results suggest that H_1-receptors could be localized to glial cells or some other non-neuronal component in the hippocampus. Localization of H_1-receptors to specific cell types remains open to speculation. Intrastriatal kainic acid injections caused a 30% decrease in mepyramine binding 4 days postlesion, which was reversed and increased to 148% of control levels by 77 days postlesion (Chang et al., 1980). This short term change was associated with a decrease in B_{max} and not antagonist affinity. H_1-receptor binding sites are also found in cortical bovine microvasculature (Peroutka et al., 1980). Prolonged treatment of guinea pigs with mepyramine did not alter the B_{max} or K_b of mepyramine binding in brain or smooth muscle (Hill et al., 1981b).

4.2. H_2-Receptor Binding Studies.

Although pharmacological characterization has revealed H_2-receptors in brain coupled to adenylate cylcase activation (see Introduction 6.2.) the identification of this HA-receptor by binding studies has proved elusive. ^{3}H-Cimetidine (Smith et al., 1980; Rising et al., 1980) or ^{3}H-HA (Palacios et al., 1978b) do not label sites with characteristics predicted from H_2-receptor activation of adenylate cyclase. One study successfully identified

H_2-receptors in guinea pig cerebral cortex using ^3H-tiotidine (Gajtkowski et al., 1983), a thiazolyl derivative with higher affinity than cimetidine for H_2-receptors (see Table 2). However, these authors and others (Maayani et al., 1982) were unable to demonstrate specific ^3H-tiotidine binding to other brain areas or tissues containing functional H_2-responses. The high degree of non-specific tiotidine binding undoubtedly contributed to this failure (Maayani et al., 1982). Recent developments of newer, highly specific, H_2-antagonists (Buyniski et al., 1984) offer hope for future development of specific H_2-receptor binding techniques.

5. H_1-Receptor Mediated Responses in Brain.

5.1. Phosphatidylinositol.

With the development of appropriate assay techniques, stimulation of phosphatidlyinositol turnover has been found in response to activation of many receptors, including the H_1-receptor (see Berridge 1984, and Berridge and Irvine, 1984 for recent reviews). This stimulation is seen in many tissues and cells, including brain.

Early studies revealed that HA-stimulated ^{32}P-incorporation (from ^{32}P-ATP) into rat brain phospholipids was mediated through H_1-receptor stimulation (Friedel and Schanberg, 1975; Subramanian et al., 1980). H_1-antagonists could block this response, whereas the H_2-antagonist cimetidine was ineffective (Friedel and Schanberg, 1975). Five min after HA administration, ^{32}P was significantly incorporated into phosphatidic acid (Subramanian et al., 1980), the parent compound of phosphoglycerides. By 30 min, HA-stimulated ^{32}P label was preferentially associated with phosphatidylinositol and phosphatidylcholine (Subramanian et al., 1980).

Recently, the B_{max} of specific high affinity ^{3}H-mepyramine binding was shown to correlate with HA-mediated increases in phosphatidylinositol turnover in different regions of guinea pig brain (as measured by increased ^{3}H-inositol-1-monophosphate formed from

^{3}H-inositol prelabeled phosphlolipids, in the presence of lithium, which inhibits inositol-1-monophosphatase) (Daum et al., 1983). Use of selective HA agonists and antagonists revealed that this HA response is mediated by H_1-receptor stimulation (Brown et al., 1984; Daum et al., 1984).

The relationship between H_1-receptor stimulation and inositol phosphate accumulation appears to vary among tissues. The presence or absence of lithium had no effect on HA-stimulated accumulation of total inositol phosphates in rat cortical slices (Brown et al., 1984). Lithium had a smaller effect on HA-stimulated ^{3}H-inositol-1-monophosphate accumulation in guinea pig cerebellar slices than in cortical and hippocampal slices (Daum et al., 1984). Whether or not this differential effect of lithium influenced the correlation between ^{3}H-mepyramine binding and HA-mediated ^{3}H-inositol-1-monophosphate accumulation was not discussed.

HA-stimulated inositol-1-monophosphate accumulation appears to be calcium-dependent. Low calcium decreased HA-stimulated (1 mM) formation of ^{3}H-inositol-phosphate in rat cerebral cortical slices (Allison et al., 1976; Hallcher et al., 1980). In another study, inclusion of EGTA (0.5 mM) or incubation in calcium-free buffer abolished HA-stimulated ^{3}H-inositol phosphate accumulation in rat cerebral cortical slices (Kendall and Nahorski,

1984). In this study, although EGTA abolished the response to all neurotransmitters examined, incubation in calcium free buffer only reduced the response to noradrenaline (100 uM) and 5-hydroxtryptamine (1 mM) and had no effect on carbachol-stimulated (100 uM) breakdown of ^{3}H-phoshatidylinositol. This finding suggests that stimulation of different hormone-receptors may cause ^{3}H-inositol-phosphate accumulation through different mechanisms. The differences in calcium and lithium dependency of hormone-receptor mediated changes in inositol phosphate accumulation may be related and suggest the possibility that the mechanism of HA-stimulated inositol phosphate accumulation may differ among brain regions and/or species.

5.2. Cyclic GMP.

Histamine stimulates cyclic GMP formation in some, but not all, nervous tissues. HA caused increases in the cyclic GMP content of mouse neuroblastoma cells (Richelson, 1978a, 1978b and 1980; Taylor and Richelson, 1979), guinea pig (Schwabe et al., 1978) and rabbit (Kuo et al., 1972) cerebral cortical slices, and rat (Lindl, 1983) or bovine sympathetic ganglia (Study and Greengard, 1978). In contrast, cerebellar slices of rabbit (Kuo et al., 1972), mouse (Ferrendelli et al., 1975) or guinea pig (Ohga and Daly, 1977) do not respond to HA with increased cyclic GMP levels.

Histamine-mediated cyclic GMP accumulation appears predominantly dependent on H_1-receptor stimulation, but H_2-receptors may also be linked to this response in some brain regions and/or species. Experiments with HA-receptor agonists and antagonists indicated that HA-mediated cyclic GMP formation in mouse neuroblastoma cells (Richelson, 1978a, 1978b and 1980) and bovine ganglia (Study and Greengard, 1978) was mediated through H_1-receptor stimulation. In rat ganglia both H_1- and H_2-receptors may be linked to cyclic GMP accumulation. Diphenhydramine (10 uM) or metiamide (10 uM) blocked HA (10 uM) stimulated cyclic GMP formation by about 50%, and full inhibition was achieved only when both H_1- and H_2-antagonists were present (Lindl, 1983).

Like other H_1-mediated responses, HA-mediated cyclic GMP formation appears to be calcium-dependent. Incubation in calcium-free medium abolishes HA-mediated cyclic GMP formation in mouse neuroblastoma cell (Taylor and Richelson, 1978). Similarly, EGTA (1-2 mM) abolishes HA-stimulated (100 uM) cyclic GMP formation in guinea pig cerebral cortical slices (Schwabe et al., 1978) and bovine superior cervical ganglion (Study and Greengard, 1978).

Until recently, it was thought that HA-stimulated increased intracellular calcium levels and that this second messenger then activated cyclic GMP formation (see

Study and Greengard, 1978). However, a recent study on mouse neuroblastoma cells suggested that arachidonic acid was the intracellular mediator for receptor-stimulated cyclic GMP formation (Snider et al., 1984). High concentrations of quinacrine (claimed to be a phospholipase A2 inhibitor by the authors) and inhibitors of lipoxygenase blocked HA-induced cyclic GMP formation. Mepyramine (1 uM) was also shown to block both cyclic GMP and the release of arachidonate formed in response to HA. This response still required calcium but it was hypothesized that formation of arachidonate, and not cyclic GMP, was the calcium-dependent step. The cyclic GMP response to HA might therefore require H_1-stimulation of phosphoinositol turnover. However, guinea pig cerebellum responds to HA with increased phosphoinositol turnover (Daum et al., 1983 and 1984) yet cyclic GMP levels do not change (Ohga and Daly, 1977). Resolution of the mechanisms whereby H_1-receptor stimulate cyclic GMP formation in brain thus require further clarification.

5.3. Glycogenolysis.

H_1-receptors stimulate breakdown of ^{3}H-glycogen in mouse cerebral cortex. The dissociation constants for H_1-antagonists on ^{3}H-mepyramine binding show a reasonable correlation with their dissociation constants derived from inhibition of HA-stimulated glycogenolysis in slices of mouse cerebral cortex (Quach et al., 1980; Schwartz et

al., 1982). This HA response was reduced by about 40% by incubation with low calcium (0.4 vs 2.6 mM) (Quach et al., 1980).

In vivo studies on chicks demonstrated HA's ability to mobilize glycogen stores by conversion of inactive phosphorylase b to phosphorylase a (Edwards et al., 1974; Nahorski et al., 1975). This HA response was, however, ascribed to H_2-receptor stimulation. Glycogenolysis was only partially blocked by metiamide and not affected by H_1-receptor antagonism (Edwards et al., 1974; Nahorski et al., 1975). Recent evidence suggests that hormone-receptor mediated glycogenolysis, although originally attributed exclusively to second messenger formation of cyclic AMP with consequent activation of cyclic AMP-dependent protein kinase and phosphorylation of phosphorylase b, may be under more complex regulatory control (see Exton, 1982). Thus, as with other receptor-mediated responses it appears likely that glycogenolysis is also subject to complex regulatory control by more than one intracellular mechanism.

6. Histamine-Mediated Cyclic AMP Accumulation in Brain.

6.1. General Considerations.

A number of different techniques have been used to characterize the HA-mediated cyclic AMP accumulation in brain, so comparison between laboratories is difficult. Studies on broken cell homogenates, using exogenous ATP as substrate are commonly conducted in nonphysiological medium containing EGTA, GTP and phosphodiesterase inhibitors. In more intact preparations such as brain slices, which are commonly maintained in physiological medium, measurements of endogenous as well as precursor-treated (labeled or unlabeled) cyclic AMP levels have been made. These latter methods often involved use of varying concentrations of different prelabeling agents (i.e. adenine and adenosine), which may have labeled different pools of ATP coupled to hormone-receptor mediated cyclic AMP accumulation (see Introduction 1.3).

With broken cell membrane preparations, the magnitude of maximal H_2-receptor stimulated adenylate cyclase activation is small, e.g. up to a doubling of cyclic AMP formation in the guinea pig hippocampus (see Daly, 1977). This is in sharp contrast to the large increases in the accumulation of cyclic AMP induced by HA in more intact preparations of brain tissue. For example, HA caused a greater than 25 fold increase in cyclic AMP levels in

rabbit cerebral cortical slices and between 7-10 fold in similar preparations from the guinea pig (Rall and Sattin, 1970). Also in contrast to broken cell preparations, this latter response has been suggested to involve both H_1- and H_2-receptors (see below). While the mechanisms underlying these differences have not been resolved, the results suggest that some form of structural integrity must be maintained in order to demonstrate H_1-receptor involvement in cyclic AMP dynamics.

As decribed below, studies of HA-induced cyclic AMP accumulation in brain differ greatly in the magnitude of the response observed and in the receptors that appear to mediate this response. While species differences may explain some of these observations, other discrepancies may be related to the different methodologies used in these studies.

6.2. Broken Cell Membrane Preparations.

6.2.1. Distribution and Localization.

Histamine stimulates brain adenylate cyclase activity in broken cell preparations from several species (see Daly, 1977; Hough and Green, 1981; Newton et al., 1982). Regional distribution studies in the guinea pig indicate that the hippocampus, cerebral cortex and corpus striatum are most responsive to HA (Hegstrand et al., 1976). Both rat and guinea pig hypothalamic preparations have been

reported to be responsive (Ahn and Makman, 1977; Huszti, 1981; Portaleone et al., 1978) or marginally responsive (Ahn and Makman, 1977; Hough and Green, 1981) to HA stimulation. The reasons for these discrepancies are unknown.

Subcellular distribution studies suggest that HA-sensitive adenylate cyclase activity in guinea pig cerebral cortex parallels the distribution of synaptic membrane fragments (Kanof et al., 1977). In the guinea pig hippocampus, the HA EC_{50} values for activation of homogenate adenylate cyclase and for increased interictal spike frequency in CA3 were both about 5 uM (Olianas et al., 1981 and 1984). These studies suggest a significant neuronal component in HA-sensitive adenylate cyclase activation. However, broken cell preparations of guinea pig (Karnushina et al., 1980) and rabbit (Palmer et al., 1980) cerebral capillary fractions, and intact human astrocytoma cells (Clark and Perkins, 1971) also possess a HA-sensitive adenylate cyclase activity.

6.2.2. Histamine Receptors Mediating Adenylate Cyclase Activation in Broken Cells.

Like other hormone-receptor interactions, HA increases the maximum velocity of adenylate cyclase without altering the K_m (0.18 mM) for the substrate MgATP (Kanof et al., 1977). Over limited concentration ranges, this HA effect

is potentiated by free magnesium and GTP and inhibited by calcium (Kanof et al., 1977).

HA-stimulated adenylate cyclase activity in broken cell membrane preparations of guinea pig hippocampus (Green et al., 1977; Kanof and Greengard, 1979), rabbit cortex (Maayani, 1982) and nucleus accumbens (Chronister et al., 1982) and probably monkey frontal cortex (Newton et al., 1982) appears to be mediated through H_2-receptors. H_2-receptor antagonists produce parallel surmountable shifts to the right in concentration-response curves to HA and dimaprit (Green et al., 1977). The slope of Schild plots derived from this data do not differ from unity, indicating that these antagonists act through classical antagonism at H_2-receptors (Green et al., 1977). Derived inhibition constants for H_2-receptor antagonists on these responses (Table 2) are similar for the effects of these compounds in inhibiting peripheral H_2-receptor mediated response such as contraction of the guinea pig atrium (Black et al., 1972; Maayani et al., 1982). All these studies argue that H_2-receptors activate adenylate cyclase in broken cell preparations of brain.

H_1-antagonists also inhibit HA-stimulated adenylate cyclase activity in broken cell membrane preparations of brain (Table 2), shifting concentration-response curves to HA and dimaprit (Maayani et al., 1982) in a parallel and

Table 2. Comparison of the apparent dissociation constants of selected H_1- and H_2-receptor antagonists on the H_2-receptor mediated stimulation of cyclic AMP formation in guinea pig hippocampus.

	Apparent dissociation constant (uM)	
Tissue preparation	Broken cell membranes*	Brain slices**
H_2-antagonists		
Metiamide	0.87 (a)	0.82, 0.91 (f,g)
Cimetidine	0.60 (a)	0.62 (f)
Tiotidine	0.03 (b)	-
H_1-antagonists		
Mepyramine	6.61 (a)	-
Chlorpheniramine	1.2 (c)	-
Promethazine	0.03 (c)	3.0 (f)
Diphenhydramine	0.55 (c)	-
Doxepin	0.15 (d)	-
Amitryptyline	0.06 (e)	3.5 (f)

Shown are the apparent dissociation constants of selected antagonists reported to inhibit H_2-receptor mediated cyclic AMP accumulation in guinea pig hippocampus. The dissociation constants shown assume that antagonists inhibit H_2-mediated cyclic AMP accumulation through classical competitive antagonism, which may not be the case for all agents (see text for further details).

* Dissociation constants reflect the ability of antagonists to inhibit H_2-mediated conversion of exogenous ^{32}P-ATP to ^{32}P-cyclic AMP.

** Dissociation constants reflect the ability of antagonists to inhibit H_2-mediated conversion of intracellular ATP (formed through adenine prelabeling) to cyclic AMP.

Data taken from (ref no.):

(a) Green et al., 1977.
(b) Maayani et al., 1982.
(c) Kanof and Greengard, 1979.
(d) Kanof and Greengard, 1978.
(e) Green and Maayani, 1977.
(f) Trung Tuong et al., 1980.
(g) Palacios et al., 1978a.

surmountable fashion. The inhibition constants of these agents, in inhibiting HA-mediated adenylate cyclase of brain (Table 2), are several orders of magnitude higher than those inhibiting H_1-receptor mediated processes, and in inhibiting ^{3}H-mepyramine binding to H_1-receptors in brain (see Table 1 and Green et al., 1977). These observations clearly indicate that classical H_1-receptor antagonism cannot explain the effects of these antagonists in this preparation of brain tissue. H_1-antagonists were therefore thought to inhibit HA-stimulated adenylate cyclase activity through competitive H_2-receptor antagonism. However, while Schild plots for mepyramine inhibition of HA, or dimaprit, stimulated adenylate cyclase activity are similar and linear over a wide antagonist concentration range, the slope of these plots are significantly less than one, indicating that this H_1-antagonist does not act through competitive antagonism at H_2-receptors (Maayani et al., 1982). The mechanism whereby H_1-antagonists inhibit H_2-receptor stimulated adenylate cyclase activity in these preparations thus remains unknown.

The rank order of potency of HA agonists in stimulating cyclic AMP accumulation has also been used to characterize HA-receptors mediating this response in broken cell membrane preparations of brain. The rank order of potency of HA agonists in stimulating

adenylate cyclase activity from homogenates of different brain regions is strikingly different. This difference can be seen in the same brain region from different species, as demonstrated for guinea pig (HA > 4-methyl-HA > 2-methyl-HA; Kanof and Greengard, 1979) and rabbit (HA > 4-methyl-HA = 2-methyl-HA; Maayani, 1982) hippocampal preparations, and in different brain regions from the same species, as in rabbit hippocampus (Mayanni, 1982) and nucleus accumbens (4-methyl-HA > 2-methyl-HA > = HA; Chronister et al., 1982). In these studies, all agonists caused the same maximum response in a given brain preparation. It is possible that alterations in H_2-stimulus-response relationships between these tissues preparations contribute to these differences in agonist profiles.

In broken cell membrane preparations of brain, high HA agonist concentrations (mM) stimulate cyclic AMP accumulation through a mechanism distinct from H_2-receptor activation. High concentrations of HA ($\geq$1 mM) stimulate adenylate cyclase activity above levels associated with maximal H_2-receptor occupancy, in broken cell membrane preparations of guinea pig hippocampus (Black et al., 1981; Maayani, 1982). This increase is not antagonized by H_1- or H_2-receptor antagonists (Maayani, 1982). Similarly, 40-50% of cyclic AMP formed in the presence of 2-(2-aminoethyl)pyridine (3 mM) (an H_1-agonist) was not

inhibited by saturating concentrations of metiamide (100 uM) or promethazine (3 uM) (Kanof and Greengard, 1979), suggesting that a non-H_2-receptor mediated component stimulates cyclic AMP accumulation in this brain preparation. Consistent with the hypothesis, 2-(2-aminoethyl)-pyridine (3 mM) stimulated adenylate cyclase activity above the levels associated with, and in the presence of, concentrations of HA (300 uM) sufficient to saturate H_2-receptors (Kanof and Greengard, 1979). The magnitude of nonspecific HA-agonist stimulated cyclic AMP levels could vary between brain regions, and should be considered as a potential complicating factor in the pharmacological classification of H_2-mediated adenylate cyclase activation. Such a possibility, could aid in explaining the different agonist properties outlined above.

In summary, a number of results demonstrate that H_2-receptors are coupled to adenylate cyclase activation in broken cell membrane preparations of brain. H_1-receptors do not appear to stimulate adenylate cyclase or alter cyclic AMP levels in this preparation. However, other properties of HA agonists may also act to increase cyclic nucleotide levels in broken cell membrane preparations of brain, as shown by different agonist properties and nonspecific HA effects. Concentration-response curves to some, or all, HA-receptor agonists could represent a

composite of two or more different actions, each of which stimulate cyclic AMP accumulation through different processes, at least in some species or brain regions.

6.3. Brain Slice and Vesicular Preparations.

6.3.1. Distribution and Localization.

In general, the species and brain regions containing a HA-responsive adenylate cyclase in broken cell membrane preparations respond similarly in brain slices and vesicular preparations (Daly, 1977). However, differences in the magnitude and mechanism of these responses, as discussed, are apparent. One exception is the monkey brain, where cortical slices appear unresponsive to HA (Forn and Krishna, 1971) but adenylate cyclase, in broken cell preparations of the same brain region, is activated by HA (Newton et al., 1982). A similar difference has been reported for dopamine-sensitive cyclic AMP stimulation in rat hypothalamus (see Ahn and Makman, 1977 for details). The reasons for these discrepancies are unknown.

As in broken cell preparations, a neuronal component may be involved in HA-sensitive cyclic AMP accumulation in brain slices. Lesions of the medial forebrain bundle result in supersensitivity of the HA-stimulated cyclic AMP accumulation in rat cortical and hippocampal slices (Dismukes et al., 1975). No changes in HA-sensitivity are found in the hippocampus after similar lesions in the guinea pig (Dismukes et al., 1976a). Kainic acid injections into the hippocampus abolished the HA-sensitive

cyclic AMP accumulation in rat hippocampal slices (Garbarg et al., 1978).

6.3.2. Histamine Receptors Mediating Cyclic AMP Accumulation.

With the advent of specific pharmacological tools to probe the HA-receptors underlying the well documented HA-induced stimulation of cyclic AMP formation in brain slices (Daly, 1977), a rather confusing picture of the receptors involved in this response has emerged. Both H_1- and H_2-receptors have been implicated in this response (see below). However, in many instances, inferences for H_1- and/or H_2-receptor participation were based on the ability of single, often inappropriate, concentrations of antagonists to inhibit the cyclic AMP response to a single saturating concentration of HA. Although the extent and nature of HA-receptors coupled to HA-stimulated cyclic AMP accumulation in brain slices is largely unresolved, it is clear that this HA response is different from that mediating adenylate cyclase activation in broken cell membrane preparations of brain (see Introduction 6.2.).

In guinea pig hippocampal slices, the cyclic AMP response to HA-receptor agonists can be eliminated by the H_2-antagonist metiamide (Palacios et al., 1978a). In this study, concentration-response curves to HA and 2-amino-ethyl-thiazole (TEA) were shifted to the right in a

parallel and surmountable fashion by metiamide. A Schild plot of the HA data gave a slope of unity, an observation compatible with the response being mediated only through H_2-receptor stimulation. While TEA is a selective H_1-agonist, the concentrations at which this agent stimulated cyclic AMP accumulation in hippocampal brain slices (EC_{50} = 200 uM) were similar to those stimulating other H_2-receptor linked processes (Green and Hough, 1980). The similarity of the apparent dissociation constant for metiamide in inhibiting HA-induced increases in cyclic AMP levels in hippocampal brain slices and broken cell homogenates (Table 2), or in inhibiting H_2-receptor mediated responses, such as atrial contraction and relaxation of the uterus (Black et al., 1972) argues that H_2-receptor occupancy underlies these HA responses. This study could therefore be interpreted to indicate that HA-mediated cyclic AMP accumulation occurs only through H_2-receptor stimulation.

H_1-receptor stimulation may also be coupled to cyclic AMP accumulation in the guinea pig hippocampal slice preparation. Low concentrations of mepyramine (3-300 nM) (Palacios et al., 1978a) and promethazine (Trung Tuong et al., 1980) (0.01 - 1 uM), inhibited the response to HA (50 uM) in a concentration-dependent manner. In both of these studies, only about 50% of the HA response was blocked by these concentrations of H_1-antagonists, with an

apparent plateau of inhibition at around 1 uM. Low concentrations of mepyramine (3-300 nM) antagonized only part of the HA concentration-response curve, which appeared to shift in a parallel and surmountable fashion with increasing concentrations of this H_1-antagonist (Palacios et al., 1978a). A Schild plot, derived from the mepyramine-sensitive portion of this HA response, gave a slope of unity and generated an apparent dissociation constant of 3.5 nM for mepyramine, a value similar to the ^3H-mepyramine binding constant on H_1-receptors in brain (Table 1). In the same study, other H_1-antagonists caused a concentration-dependent inhibition of the cyclic AMP response to a fixed concentration of HA (100 uM), and confirmed that only 50% of the HA response was blocked by these agents (Palacios et al., 1978a). The authors concluded that the dissociation constants for H_1-antagonists in inhibiting this HA response were in agreement with those determined from inhibition of HA-induced contraction of the guinea pig ileum, an H_1-receptor mediated response (see Table 1 and Palacios et al., 1978a). However, as noted (Johnson, 1982), the correlation between these two measures is poor. Thus, while it is probable that an H_1-receptor mediates both of these HA responses, further pharmacological characterization of these responses are required to explain this anomaly.

The cyclic AMP response to HA in guinea pig hippocampal slice preparations not inhibited by lower concentrations of H_1-antagonists may be mediated through H_2-receptors. At higher concentrations (>10uM), after a plateau (see above), the H_1-antagonists promethazine and cyproheptidine inhibited the remaining HA-induced activity (Trung Tuong et al., 1980). Concentration-response curves to the H_2-agonists dimaprit and impromidine were shifted to the right in a parallel and surmountable fashion by promethazine (8 and 10 uM respectively). Derived K_i values for promethazine in inhibiting these H_2-receptor mediated responses were 5 - 6 uM. These values are approximately two orders of magnitude higher than those obtained in similar studies of broken cell preparations (see Table 2).

It has been suggested that, after studies of guinea pig hippocampal slices, H_2-receptor activation is a prerequisite to the appearance of an H_1-receptor involvement in the overall cyclic AMP response to HA (Palacios et al., 1978a). This hypothesis was supported by the observation that the concentration-response curve to the selective H_1-agonist TEA was shifted to the left in the presence of a saturating concentration (100 uM) of the selective H_2-agonist dimaprit (Palacios et al., 1978a). In the presence of dimaprit (100 uM), the response to TEA was antagonized by the H_1-antagonist

mepyramine (Ø.1 uM) (Palacios et al., 1978a). Thus, in the absence of dimaprit the $EC_{5Ø}$ (2ØØ uM) of the response to TEA reflected a requirement of TEA to activate H_2-receptors before an H_1-component in the response to this agonist could be measured, while in the presence of dimaprit the ability of TEA ($EC_{5Ø}$ = 6Ø uM) to stimulate H_1-receptors could now be seen.

Although HA also stimulates cyclic AMP accumulation in guinea pig cortical slices, the distribution and/or effector mechanisms governing this response may differ from that found in hippocampal tissue. Conflicting reports on the nature of HA-receptors mediating cyclic AMP accumulation in guinea pig cerebral cortex have appeared. Several studies have shown that the cyclic AMP response to HA is abolished by high concentrations (1-1Ø uM) of H_1-antagonists (Chasin et al., 1973; Hill et al., 1981a) suggesting that only H_1-receptors mediate the response in this tissue. This contention was supported by the observation that cimetidine (1ØØ uM) could not inhibit the HA response (1ØØ uM) and that dimaprit (1 mM) had no or very small effects on cyclic AMP levels (Hill et al., 1981a). In contrast, other laboratories have shown that about 5Ø% of the response to HA can be blocked by either H_1- or H_2-antagonists and that a combination of these agents was required to abolish the HA response (Baudry et al., 1975; Rogers et al., 1975). One study reported a

small response to the H_2-agonist 4-methyl HA (Dismukes et al., 1976b) while another demonstrated a substantial response to this agonist which was abolished by an H_2- and not an H_1-receptor antagonist (Baudry et al, 1975). It is possible that these discrepances can be attributed to differences in methodologies employed by the different investigators.

Interestingly, the properties of HA-receptors mediating cyclic AMP accumulation in rabbit cerebral cortical slices are similar to those found in guinea pig hippocampus. In rabbit cortical slices (Al-Gadi and Hill, 1984), the entire response to HA was blocked by the H_2-antagonist cimetidine (K_i = 1.5 +/- Ø.3 uM). In this study, the H_1-antagonist mepyramine (1 uM) only inhibited 5Ø% of the HA response, causing a surmountable shift of the upper half of the HA response of about an order of magnitude. This suggests that the mechanism whereby H_1- and H_2-receptors interact to cause cyclic AMP formation may be similar in rabbits and guinea pigs.

Preliminary evidence suggests that H_1- and H_2-receptors may also be coupled to ^{3}H-cyclic AMP accumulation in a vesicular ('synaptosomal-like') preparation of brain. In guinea pig cortical and hippocampal vesicular preparations, the ^{3}H-cyclic AMP response to 2-methyl-HA (1ØØ uM) was inhibited by both the H_1-antagonist tripelennamine (Ø.1 - 1.Ø uM) and metiamide

(1 and 10 uM) while only metiamide inhibited the response to 4-methyl-HA (100 uM) (Psychoyos, 1978). In a similar study, brompheniramine (1 uM) inhibited HA-induced (100 uM) ^{3}H-cyclic AMP accumulation in a vesicular preparation of guinea pig cortex (Daly et al., 1980). Although pharmacological characterization of these HA responses have not been reported, it seems probable that the relationship between HA-receptors increasing cyclic AMP accumulation in brain slices may be maintained, at least qualitatively, in the vesicular preparation.

6.3.3. Adenosine Involvement in the Cyclic AMP Response to Histamine.

Exogenous adenosine appears to act synergistically with HA, and other neurohormones, on cyclic AMP formation in brain slices (Daly, 1977). This effect is believed to be mediated through the stimulation of an extracellular adenosine receptor coupled to cyclic AMP accumulation (see Introduction 2.).

It has been suggested that exogenous adenosine potentiates the response to H_1-receptor stimulation (Dismukes et al., 1976b). In guinea pig hippocampal slices, TEA (100 uM) and 4-methyl-HA (100 uM) elicited accumulations of ^{14}C-cyclic AMP in both the presence and absence of adenosine (Dismukes et el., 1976b). In the presence of adenosine (100 uM) the response to both

agonists increased (Dismukes et al., 1976b). In this study, the response to TEA was antagonized by both d-brompheniramine (10 uM) (an H_1-antagonist) and metiamide (10 uM) in the absence of exogenous adenosine, while in its presence only d-brompheniramine was an effective antagonist. Conversely, the response to 4-methyl-HA was antagonized only by metiamide in the absence of adenosine, while in its presence both H_1- and H_2-antagonists were effective. The concentration-response curve to the H_1-agonist TEA (EC_{50} = 250 vs 24 uM), but not HA (EC_{50} = 12 uM), was shifted to the right by adenosine (100 uM) (Dismukes et al., 1976b). These observations, coupled with those of Palacios et al. (1978a), suggest that H_1-mediated accumulation of cyclic AMP in guinea pig hippocampus is potentiated by either adenosine or H_2-receptor stimulation.

Adenosine also potentiated the response to HA in both guinea pig (Daum et al., 1982; Hill et al., 1981a) and rabbit (Al-Gadi and Hill, 1984) cerebral cortical slices. In the presence of adenosine (0.1 mM) HA and TEA produced a 3-15 fold rise in cyclic AMP in guinea pig cerebral cortical slices (Hill and Young, 1981). This HA response was inhibited by H_1-antagonists but not by the H_2-antagonist cimetidine (0.1 mM) (Hill and Young, 1981). The affinity constants of H_1-antagonists in inhibiting the HA-potentiation of the response to adenosine (100 uM) were

in good agreement with values obtained from inhibition of ^{3}H-mepyramine binding (see Table 1 and Hill et al., 1981a), strongly suggesting that this response was mediated through H_1-receptor stimulation. In rabbit cerebral cortical slices, addition of exogenous adenosine (100 uM) increased the mepyramine-sensitivity of the cyclic AMP response to HA (Al-Gadi and Hill, 1984). In the same study, cimetidine nolonger inhibited the entire HA response. Analysis of the cimetidine-sensitive component of this response generated a K_i value (1.1 uM) similar to that obtained in the absence of exogenous adenosine. As in guinea pig hippocampus, all the evidence cited above appears to demonstrate that H_1-mediated cyclic AMP accumulation in cerebral cortex, requires either adenosine or H_2-receptor stimulation.

Endogenous adenosine, formed during incubation of brain slices (Shimizu et al., 1970; Hill et al., 1981a), synaptosomes (Kobayashi et al., 1981) and vesicular preparations (McNeal et al., 1980) may also influence the overall response to HA. Preincubation with adenosine has been shown to cause a substantial decrease in the subsequent response to HA in brain slices (Schultz and Daly, 1973c). Hill et al. (1981a) demonstrated that adenosine deaminase (1.6 U/ml), which converts adenosine to its inactive metabolite inosine, substantially reduced both basal and HA-stimulated (100 uM) cyclic AMP

accumulation. In addition to previously cited evidence, this latter finding suggests that HA-mediated cyclic AMP accumulation in this preparation of guinea pig cortical slices is predominantly dependent on adenosine. In contrast, theophylline (300 uM), at a concentration sufficient to block adenosine-mediated cyclic AMP accumulation, did not inhibit HA-stimulated (100 uM) ^{14}C-cyclic AMP accumulation in guinea pig cerebral cortical slices (Rogers et al., 1975). Interestingly, (in the absence of theophylline) this latter study also demonstrated an H_2-receptor involvement in the HA response (see above). McNeal et al (1980), demonstrated that preincubation with adenosine deaminase (10 ug/ml), prevented a time-dependent decline in both basal and HA-stimulated ^{3}H-cyclic AMP levels in a guinea pig cortical vesicular preparation. These authors also found that the maximum response to HA (100 uM) was decreased by 60% in the continued presence of adenosine deaminase. Pretreatment with adenosine deaminase also reduced basal activity (total cellular cyclic AMP) in a synaptosomal preparation from rat cerebral cortex (Kobayayashi et al., 1981).

6.3.4. Calcium Involvement in the Cyclic AMP Response to Histamine.

Calcium also appears to be involved in regulating HA-mediated cyclic AMP accumulation in brain. However, it is unclear that H_1-mediated cyclic AMP accumulation is completely calcium dependent. Consistent with a role of calcium in H_1-mediated cyclic AMP accumulation, in the presence of EGTA (2 mM) and theophylline (200 uM), the ^{14}C-cyclic AMP response to HA (100 uM) in guinea pig cortical slices was not significantly inhibited by the H_1-antagonist brompheniramine (100 uM), but was eliminated by the H_2-antagonist metiamide (100 uM) (Daly et al., 1979). In another study, the cyclic AMP response to dimaprit (100 uM) was unchanged by incubation in calcium-free buffer, while the response to a combination of dimaprit (100 uM) and TEA (100 uM) was reduced compared to incubation in the presence of calcium (2.6 mM) (Schwartz et al., 1980a). However, the response to TEA and dimaprit was still higher than the response to dimaprit alone, suggesting that TEA still increased cyclic AMP accumulation, possibly through H_1-receptor stimulation. While neither of these studies are definitive, they are compatible with the hypothesis that H_1-mediated cyclic AMP accumulation is regulated by calcium.

Preliminary evidence suggests that H_1-receptor stimulated cyclic AMP accumulation in brain may not always

require calcium. In the presence of EGTA (2 mM), but the absence of theophylline, approximately 50% of HA-stimulated (100 uM) ^{14}C-cyclic AMP accumulation was blocked by either brompheniramine (100 uM) or metiamide (100 uM). In guinea pig cerebral cortical slices EGTA (2 mM) had no effect on the maximum response to HA (100 uM) while a combination of EGTA and adenosine deaminase (10 ug/ml) abolished the HA response (Schwabe et al., 1978). In both these studies, EGTA increased basal activity, an effect suggested to reflect increased adenosine release, with consequent increased cyclic AMP accumulation due to adenosine receptor stimulation (Schwabe et al., 1978). It appears likely that adenosine selectively potentiates the response to H_1-receptor stimulation (see Introduction 6.3.3.). Therefore, in the presence of adenosine, H_1-receptor mediated cyclic AMP accumulation might be elicited in a calcium independent fashion.

In addition to stimulation of cyclic AMP accumulation, H_1-receptors are also associated with increased formation of cyclic GMP, glycogenolysis and increased phosphatidylinositol turnover (see Introduction 5). One or more of these latter responses may mediate H_1-receptor stimulated cyclic AMP accumulation. Calcium may act as common regulator and/or mediator of all of these H_1-responses.

7. Rationale and Aims of This Study.

It has long been recognized that understanding neurohumoral regulation of cyclic AMP is hampered by the lack of suitable techniques for preparing cellular CNS fractions which retain biological activity (e.g. Sattin and Rall, 1970). Use of broken cell membrane preparations has revealed several mechanisms of action at hormone receptors associated with the activation or inhibition of adenylate cyclase (see Introduction 1.2.). However, many agents which cause large changes in cyclic AMP levels in brain slices have no effect in broken cell preparations. In the presence of HA, H_2-receptors appear to be coupled to adenylate cyclase activation in broken cell membrane preparations (see Introduction 6.2.), yet both H_1- and H_2-receptors have been implicated in HA-mediated cyclic AMP accumulation in brain slice preparations (see Introduction 6.3.).

In brain slice studies, interpretation of hormone-receptor mediated changes in cyclic AMP is complicated by potential cell-to-cell interactions and diffusional constraints. These factors, in addition to the poor experimental design commonly used in studies of HA-mediated cyclic AMP accumulation in brain, contribute to the current confusion on the relationship between HA-receptor(s) stimulation and this response. A brain preparation in which permeability barriers are removed but

that retains hormonally-responsive cyclic AMP generating systems was required. Such a preparation consisting of vesicular entities (membrane-bound synaptosomal-like structures) obtained by homogenization of brain tissue in Krebs-Ringer bicarbonate medium, was developed by Chasin et al. 1974, but has not been extensively utilized.

Studies on hormone-responsive cyclic AMP generating systems in the vesicular preparation have practical advantages over both brain slice and broken cell preparations. In particular, the ability to control the concentration of drugs at their receptor(s) would eliminate a major complicating factor in the interpretation of receptor-mediated changes in cyclic AMP. In addition, comparative studies between different CNS preparations would allow formulation of hypotheses on the likely mechanism(s) and/or sites of action of neurohumoral-sensitive cyclic AMP generating systems in the CNS.

In this thesis, the suitability of the vesicular preparation as an in vitro model for neurohumoral-mediated changes in cyclic AMP was investigated through studies on HA-receptors coupled to ^{3}H-cyclic AMP accumulation (formed by prelabeling intracellular ATP with ^{3}H-adenine) in a vesicular preparation of guinea pig cortex.

Thus the overall goals of this study were:

1. To develop incubation conditions that provide reproducible, stable ^{3}H-cyclic AMP responses to histamine in the vesicular preparation.

Reports of variability and decline in both basal and hormone stimulated ^{3}H-cyclic AMP accumulation during incubation of vesicular preparations have limited the use of this preparation (Daly et al., 1980; Chasin et al., 1974; Shimizu et al., 1975). Stabilization of basal and HA-stimulated ^{3}H-cyclic AMP accumulation was therefore sought through alterations in reported methodologies. Since a goal of this study was to characterize pharmacologically this HA response, a stable response was particularly important in this study.

2. To characterize pharmacologically the histamine-receptor(s) mediating histamine-stimulated ^{3}H-cyclic AMP accumulation in the vesicular preparation.

As discussed, only H_2-receptors appear to be coupled to adenylate cyclase activation in broken cell membrane preparations, yet both H_1- and H_2-receptors have been implicated in brain slice studies. It was therefore of interest to determine whether cellular disruption in physiological medium was sufficient to eliminate the H_1-component in the cyclic AMP response to HA.

Assuming the H_1-component was maintained, it was of interest to compare the pharmacological characteristics of H_1-receptor mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation with other H_1-mediated responses. The correlation between the reported dissociation constants for H_1-antagonists in inhibiting H_1-receptor stimulated cyclic AMP accumulation in guinea pig hippocampal brain slices compared to contraction of the isolated guinea pig ileum is poor (Johnson, 1982). It is unclear whether this discrepancy reflects tissue differences, e.g. the dissociation constants of H_1-antagonists were influenced by differences in the ability of these agents to reach their site(s) of action, or if the H_1-receptors coupled to these responses exhibit different pharmacological specificities. Determination of H_1-antagonist dissociation constants in the vesicular preparation, in which diffusional constraints are presumably removed, would aid in resolving this disparity.

With these aims in mind, all antagonists used in these studies were selected on the basis of their abilities to inhibit the cyclic AMP response to HA in both broken cell and slice preparations of brain.

2a. To determine the effect of endogenous adenosine on histamine-stimulated ^{3}H-cyclic AMP accumulation in the vesicular preparation.

Adenosine had been reported to increase H_1-receptor involvement in HA-mediated cyclic AMP accumulation from guinea pig cortical slices and is present in the incubation medium of the vesicular preparation (see Introduction 6.3.3.). It was therefore of interest to investigate whether sufficient adenosine was present in the incubation medium to influence HA-responsive ^{3}H-cyclic AMP accumulation in the vesicular preparation, and the nature of this influence, if any, on HA-receptors mediating this response. In some studies, adenosine deaminase (2.5 U/ml), which metabolizes adenosine to its inactive metabolite inosine, was therefore included in the incubation medium, and the HA-receptors mediating HA-stimulated ^{3}H-cyclic AMP accumulation under these conditions were studied.

2b. To determine the influence of calcium on histamine-stimulated ^{3}H-cyclic AMP accumulation in the vesicular preparation.

Preliminary evidence had suggested that calcium might be required for H_1-mediated cyclic AMP accumulation in brain slice studies (see Introduction 6.3.4.). To determine whether any components of the HA response were

also calcium-dependent, the HA-receptors coupled to ^{3}H-cyclic AMP accumulation in the presence of EGTA (2 mM) and adenosine deaminase (2.5 U/ml) were characterized. Adenosine deaminase was included in these studies to preclude potential complexities incurred by calcium reduction on adenosine and HA-mediated responses (see Introduction 6.3.4.).

3. <u>To develop a model to explain the pharmacological characteristics of HA-mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation.</u>

In brain slice studies, it had been suggested that H_1-receptor mediated cyclic AMP accumulation required either the presence of adenosine or concommitant H_2-receptor stimulation (Palacios et al., 1978a; Daly et al., 1980). Assuming a similar relationship was found to occur in the vesicular preparation, determination of parameters (e.g. antagonist dissociation constants) from the pharmacological characteristics of this HA response required development of an appropriate model. Fitting the pharmacological data to this model would permit comparison of these derived parameters with other H_1- or H_2-mediated responses. In addition, deviations of the pharmacological data from the predictions of the model, if any, might bear on the mechanisms whereby HA-receptors stimulate cyclic AMP accumulation in brain.

METHODS

1. Materials.

Male Hartley Albino guinea pigs (400-450 g; Perfection Breeders, Douglassville, PA) were used in all experiments.

The following drugs were generously donated: dimaprit dihydrochloride, 2-thiazolylethylamine dihydrochloride, cimetidine and metiamide (Smith Kline and French, Welwyn Garden City, England); tiotidine (ICI, Philadelphia, PA); doxepin hydrochloride (Pfizer, Brooklyn, NY); amitriptyline hydrochloride, d- and l-chlorpheniramine maleate (Merck, Sharp and Dohme, West Point, PA).

Compounds obtained from commercial sources were: histamine dihydrochloride, mepyramine (pyrilamine) maleate, promethazine, diphenhydramine and adenosine deaminase Type VII (Sigma Chemical Co.); [2-^{3}H] adenine and [8-^{14}C] cyclic AMP were from New England Nuclear (Boston, MA); all other reagent grade compounds were from standard commercial sources.

Borosilicate glass scintillation vials (20 ml) from Kimble (Toledo, OH); conical (1.5 ml) and round-bottomed (13 ml) polyproplyene test tubes from Sarstedt (Princeton, NJ); polyproplyene columns with plastic filter discs from Isolab (Akron, OH).

2. Composition of Krebs-Ringer Bicarbonate Buffers.

All brain homogenates and test agents were made up in modified Kreb's-Ringer-bicarbonate (KRB) buffers, continuously equilibrated with 95% O_2- 5% CO_2. A series of 4 buffers were studied (Table 1). These were designated 25 mM KRB, 15 mM KRB, phosphate or hepes. The approximate osmolarity of all buffers was 300-320 mOsm. For any given experiment the same buffer was used throughout preparative and incubation procedures.

3. Preparation of Brain Vesicles.

Guinea pigs were decapitated and the brain rapidly removed and placed on an ice plate. Cerebral cortices were dissected out, striatal matter was removed and the cortices placed in cold buffer for 5 min. Each cortical hemisphere was then chopped (1 x 1 mm) on a McIlwain mechanical tissue chopper, manually homogenized in 1 ml of cold buffer with 4 strokes in a Dounce homogenizer (7.11 - 11.94 mm clearance) then decanted into 13 ml polypropylene test tubes (95 x 16.8 mm). Both pestle and vessel were rinsed with 1 ml buffer, which was added to the homogenate sample. This procedure was repeated for each cortical hemisphere. The homogenates (approximately 3 Vol (original wt.)) were centrifuged (1,000 x g for 15 min at 4°C) and the supernants discarded. The vesicular preparation was obtained by gentle resuspension of the

Table. 3 Composition of Krebs-Ringer bicarbonate buffers.

Buffer Name	Final Concentration (mM)				
	NaCl	$NaHCO_3$	Na_2HPO_4	NaH_2PO_4	Hepes
25 mM KRB	112	25	-	-	-
15 mM KRB	122	15	-	-	-
Phosphate	128.34	4	2.1	0.46	-
Hepes*	125	4	-	-	25

The composition of buffers used in the present study are shown. All buffers additionally contained 3.0 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 10 mM dextrose and 10 mM sodium pyruvate. Total Na^+ concentration was maintained at 147 mM in all buffers.

* Hepes buffer was prepared from 250 mM stock, which was adjusted with NaOH to pH 7.4 at room temperature. This contributed 8 mM to the final Na^+ concentration.

pellets in 5 or 15 Vol (original wt.) of cold buffer (see below).

4. Incubation of Brain Vesicles.

Two methods were studied to label intracellular nucleotide pools: i) bulk ^{3}H-adenine-labeling of the 5 Vol preparation, or ii) individual ^{3}H-adenine-labeling of aliquots of the 15 Vol preparation.

4.1. Bulk ^{3}H-Adenine Labeling.

Unless otherwise specified, bulk-labeled preparations were preincubated at 37°C with shaking (1 cycle/sec) for 40 min in 50 ml erlenmeyer flasks under a continuous stream of 95% O_2- 5% CO_2. ^{3}H-adenine was then introduced to the 5 Vol preparation such that its specific activity was 10 Ci/mmol at 0.5 uM and then incubated a further 25-35 min. Aliquots (0.2 ml) of the ^{3}H-labeled suspension were transferred at fixed time intervals to glass scintillation vials containing 0.75 ml of pre-gassed buffer at 37°C. The air space was flushed with 95% O_2-5% CO_2, vials were sealed (with plastic liners) and incubated with rapid shaking (3 cycles/sec) for a further 20 min.

4.2. Individual ^{3}H-Adenine Labeling.

The suspension (0.7 ml of the 15 Vol preparation) was distributed at 15 sec intervals to scintillation vials,

flushed with 95% O_2-5% CO_2, sealed, and preincubated for 40 min at 37°C, with mixing (1 cycle/sec) and regassing at 20 min. ^{3}H-adenine (1 uCi/50 ul) was added to each vial to obtain a specific activity of 2.67 Ci/mmol at 0.5 uM (in 0.75 ml) and this mixture was incubated for 25 min. Fresh buffer or test agents (0.2 ml) were added, the vials were flushed with 95% O_2-5% CO_2, and the incubation continued for a further 20 min with rapid shaking (3 cycles/sec).

Following these labeling protocols, stimulation of ^{3}H-cyclic AMP formation was initiated with the addition of histamine (HA) or other test agents, in 50 ul of buffer. This final 10 min incubation (1.0 ml) was conducted under air except for incubation in 15 mM KRB which was under 95% O_2-5% CO_2.

Replicate determinations (duplicate or triplicate) were determined consecutively. First replicate determinations, for all HA concentrations (1 - 1000 uM) were run before second replicates etc.. The order of addition of different HA concentrations was randomized between experiments (but not between replicates).

Incubations (10 min) were terminated with the addition of 0.1 ml 72% trichloroacetic acid (TCA) and samples then transferred to ice.

The methodologies described above were utilized in developing a stable vesicular preparation from which pharmacological characterization of HA-sensitive ^{3}H-cyclic AMP could be reliably assessed (see Section I results). All pharmacological experiments (Section II results) were conducted in 15 mM KRB using the individual labeling protocol described above. This incubation protocol is summarized in Fig. 1.

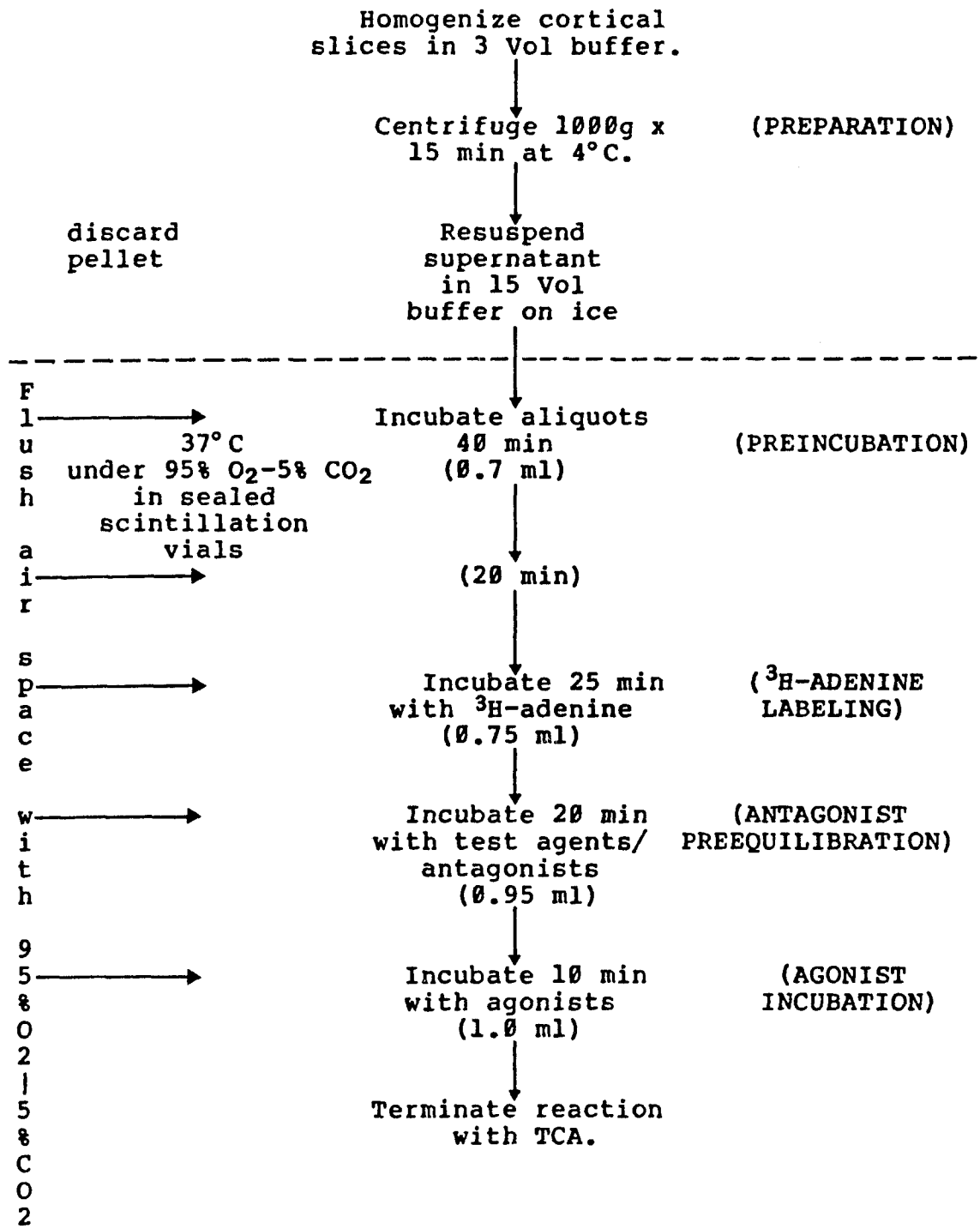
5. Isolation of ^{3}H-Cyclic AMP.

A Ø.1 ml solution of ^{14}C-cyclic AMP (approximately 2ØØØ dpm) containing unlabeled cyclic AMP (Ø.18 mM) and ATP (1.2 mM) in H_2O was added to all samples. Samples were mixed, centrifuged (2Ø,ØØØ x g for 5 min at 4 C) and supernatants received Ø.1 ml of 1% sodium lauryl sulphate (SDS).

Cyclic AMP was isolated by a procedure based on the method of Salomon et al (1974) (Fig. 2). In brief, each supernatant was passed through AG 5ØW-X4 cation exchange resin (Biorad; H^{+}-form; 2 ml hydrated resin bed) in polyproplene columns (1Ø mm internal diameter) followed by 2 x 2 ml H_2O. In some experiments this latter fraction was collected for estimation of ^{3}H-nucleotide content (ATP and ADP; Krishna et al, 1968). The next 3 x 2 ml H_2O effluents, containing the major portion of cyclic AMP, were collected onto alumina oxide columns (2 ml resin

Fig. 1. Flow diagram of the preparation, individual ^{3}H-adenine labeling and incubation of the cortical vesicular preparation.

Shown is a flow diagram of the various stages (labeled in caps far right) of the preparation and individual incubation of aliquots of the guinea pig cortical vesicular preparation. For each step, the total volume in each vial is given in parentheses. Vials were flushed with 95% O_2-5% CO_2 at the times shown by arrows. This protocol was followed in all pharmacological studies, where preparation and incubation was in 15 mM KRB (see Results 2). Further details are given in the text.


Homogenize cortical slices in 3 Vol buffer.
Centrifuge 1000g x 15 min at 4°C.
(PREPARATION)
discard pellet
Resuspend supernatant in 15 Vol buffer on ice
Flush air space with 95% O2-5% CO2
37°C under 95% O2-5% CO2 in sealed scintillation vials
Incubate aliquots 40 min (0.7 ml)
(PREINCUBATION)
(20 min)
Incubate 25 min with 3H-adenine (0.75 ml)
(3H-ADENINE LABELING)
Incubate 20 min with test agents/ antagonists (0.95 ml)
(ANTAGONIST PREEQUILIBRATION)
Incubate 10 min with agonists (1.0 ml)
(AGONIST INCUBATION)
Terminate reaction with TCA.

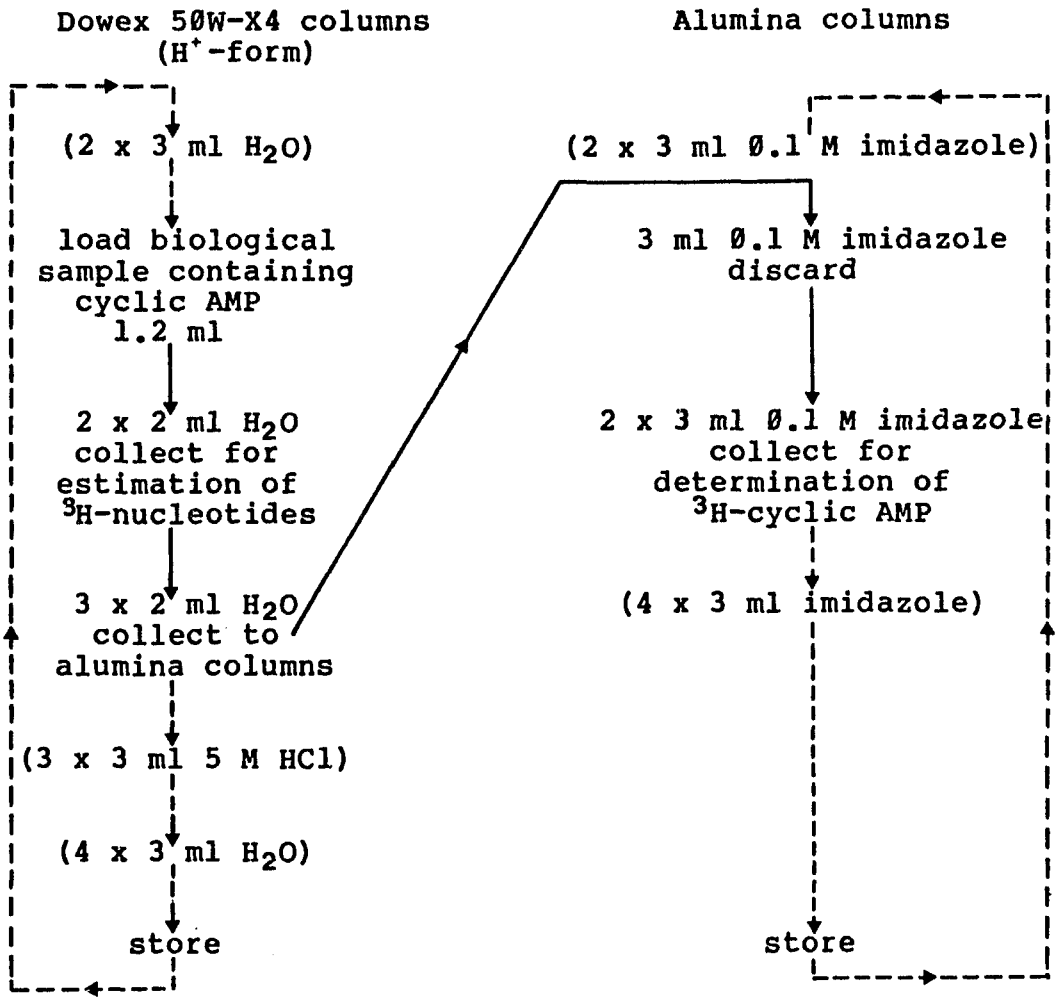


Fig. 2 Flow diagram of the isolation of ^{3}H-cyclic AMP by column chromatography.

Shown is a schematic representation of the various stages used in the isolation of ^{3}H-cyclic AMP from other labeled compounds by column chromatography. The volumes indicate the amount of buffer or H_2O added to each column at that step.

—— = elution protocol

.... = wash and regeneration cycle

See text for further details.

bed). Alumina columns were washed with 3 ml Ø.1 M imidazole buffer (pH 7.5) and the following 2 x 3 ml imidazole effluent fractions* were collected into glass scintillation vials.

Ten ml of New England Nuclear 243 scintillation cocktail was added and radioactivity determined in a scintillation counter (Beckman LS38Ø1). A discriminator setting was used to exclude spill from the ^{3}H into the ^{14}C channel. All ^{3}H-cyclic AMP values were corrected for ^{14}C spill into the ^{3}H channel and for ^{3}H counting efficiency (approximately 25%). In separate experiments ^{14}C-cyclic AMP recoveries ranged from 55-65%. Within the same experiment, differences in recoveries were routinely around 1%.

6. Protein Determination.

TCA protein pellets were solubilized overnight at room temperature in 1 ml 1Ø% SDS. Three ml H_2O were added, and protein content was estimated from ultraviolet absorption at 28Ø nm, corrected for interference by nucleic acids by reading at 26Ø nm (Layne, 1957). Values determined by this method were not significantly different from values determined by a modified Lowry procedure (Markwell et al.,

* Thin layer chromatography of these fractions on silica gel-GF in n-butanol-methanol-ethyl acetate-ammonium hydroxide (7:3:4:4, by vol.) gave a single radioactive peak at RF Ø.65, identical to that observed for unlabeled cyclic AMP vizualized under UV wavelength and similar to reported values (Shimizu et al., 1969).

1978). Final protein concentration fell between 2-3 mg/ml under all experimental conditions, and varied less than 10% within a given experiment.

7. Data Evaluation.

Results are expressed as either absolute agonist stimulation (total ^{3}H-cyclic AMP minus basal ^{3}H-cyclic AMP) or fold stimulation (absolute stimulation/basal). Duplicate or triplicate data points were obtained in each experiment.

Agonist concentration-response curves were fitted by an iterative least-squares fit to a form of the logistic function (Parker and Waud, 1971; Johnson, 1979):

$$E = \frac{E_{max}}{[1 + (EC_{50}/[A])^n]}$$

[A]= agonist concentration; E = absolute agonist stimulation; E_{max} = maximum agonist stimulation; EC_{50} = [A] producing 50% E_{max}; n = slope index (a parameter that describes the steepness of the curve.) Unless otherwise stated, the logistic fit was performed for each experiment using absolute mean data points obtained from triplicate determinations.

In some studies, additional analysis was performed by the computerized, nonlinear least-squares curve-fitting procedure FITFUN (Baig and Reid-Miller, 1980). The best

fit of the model to the data was assessed by comparing the residual sum of squares of the fitted curves at equal degrees of freedom (an index indicating the extent of deviation of the fitted values from the data) (Baig and Reid-Miller, 1980). Additional information on the models and the fitting is presented in the Results and Discussion sections.

A partial F-test (see De Lean et al., 1978; and Burgisser, 1983) was used to determine the parallelism of concentration-response curves in the presence and absence of H_2-receptor antagonists. This was done by simultaneously fitting the set of curves to logistic functions with the slope indices either being allowed to vary (variable slope) or being constrained to the same value (common slope). If the more complex variable slope model provided a better fit of the data, it was concluded that the curves were not parallel.

The dissociation constants for H_2-antagonists were determined from a Schild plot (Arunlakshana and Schild, 1959; Tallarida et al., 1979). If the slope of the plot was not significantly different from 1.0, the intercept of a line where the slope was constrained to 1 was used in determining pA_2 values (Waud and Parker, 1971; Tallarida et al., 1979).

All values are expressed as arithmetic means +/- SEM, except EC_{50} values, which are expressed as geometric means +/- SEM or 95% confidence limits, (Fleming et al., 1972; De Lean et al., 1982).

Parametric statistical tests were performed on normally-distributed data. Levels of significance were calculated using appropriate parametric statistical tests for normally-distributed data (see text for specific tests used). One way analysis of variance (ANOVA), if significant, was followed by the Newman-Keuls multiple range test (NK). Values were considered not significantly different if P-values were >0.05 and, in general, only P-values <0.05 are quoted.

Data analysis was done on the PROPHET computer system, a national resource supported by the Chemical-Biological Information Handling Program, Division of Research Resources, National Institutes of Health.

RESULTS

1. Methodological Developments.

1.1. Influence of Preincubation Time on ^{3}H-Cyclic AMP Levels.

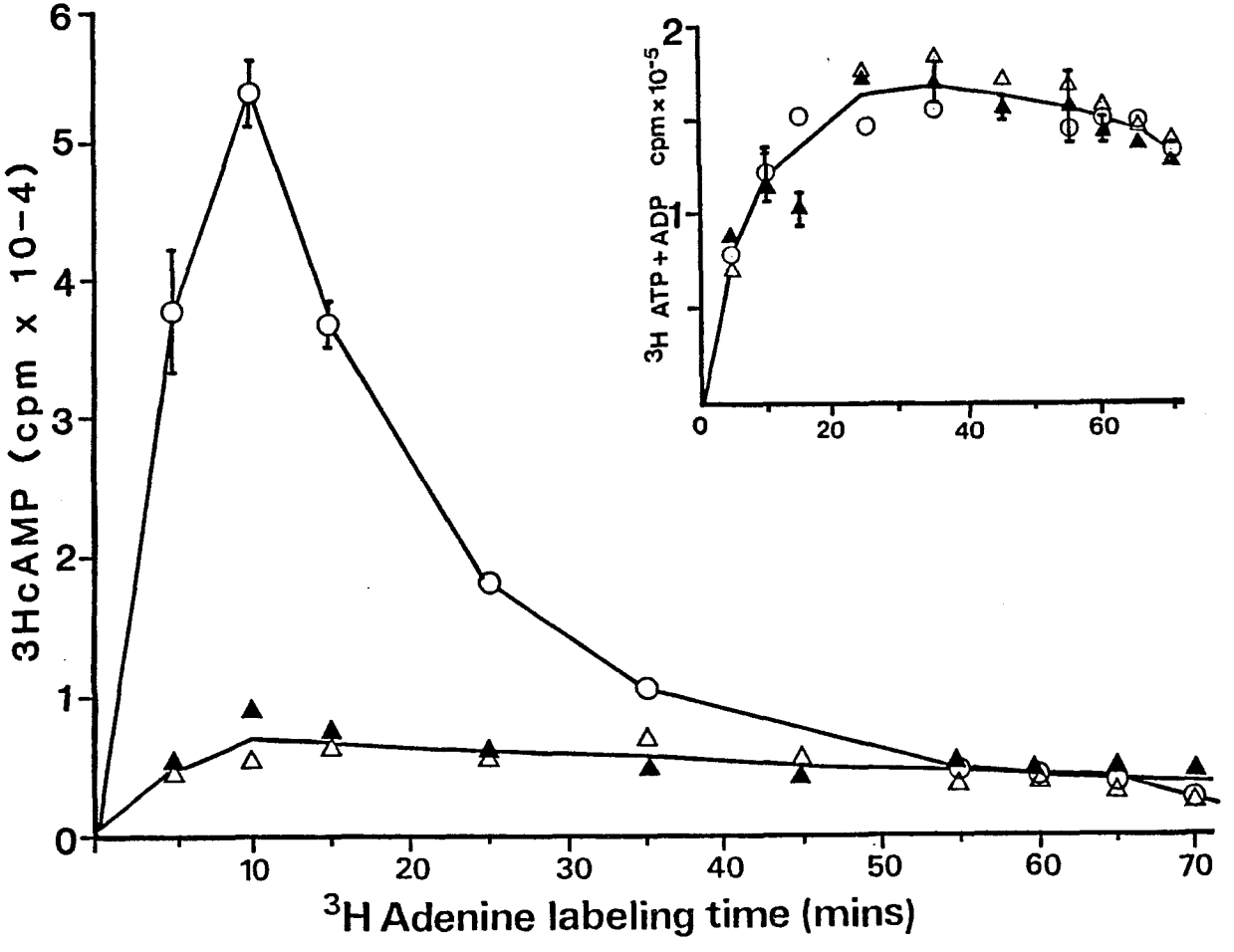
Variation in the preincubation time prior to bulk ^{3}H-adenine labeling caused pronounced changes in the pattern of ^{3}H-cyclic AMP formation (Fig. 3), with no change in ^{3}H-nucleotide formation (Fig. 3 insert). A large transient rise and subsequent decline in ^{3}H-cyclic AMP levels occurred after vesicular preparations were preincubated for 15 min prior to the addition of ^{3}H-adenine. Increasing the preincubation time to 40 or 60 min largely eliminated this flux. With increased ^{3}H-adenine labeling times (i.e. > 15 min) ^{3}H-cyclic AMP levels declined in all preparations, but the decline in 40 or 60 min preincubated preparations was smaller than in 15 min preincubated preparations (Fig. 3). ^{3}H-cyclic AMP levels were similar for all preincubation times studied when HA (300 uM for 10 min) stimulation was initiated at 45 min after addition of ^{3}H-adenine (not shown).

^{3}H-nucleotide levels, an index to ^{3}H-adenine uptake and the ^{3}H-cyclic AMP precursor pool, were uninfluenced by preincubation time (Fig. 3. inset) or HA-stimulation (not shown). ^{3}H-nucleotide formation was rapid, reaching an apparent steady-state in less than 25 min of ^{3}H-adenine

Fig. 3. Effect of preincubation time on the formation of ^{3}H-cyclic AMP.

Three guinea pig cortices were homogenized, centrifuged and resuspended in 5 Vol of 25 mM KRB. The preparation was divided equally into 3 flasks and incubated at 37°C for 15 (○), 40 (▲) or 60 (△) min. ^{3}H-adenine was added and incubation in bulk continued for a further 15 min. Aliquots (0.2 ml) were then distributed to scintillation vials containing 0.75 ml of prewarmed buffer and incubation under 95% O_2-5% CO_2 continued with rapid shaking (3 cycles/sec). Values shown represent the mean +/- SEM of triplicate determinations of ^{3}H-cyclic AMP levels determined after various elapsed times following ^{3}H-adenine addition. The experiment was repeated with similar results.

Inset:- Effect of preincubation time on formation of ^{3}H-nucleotides (ATP + ADP). Counts shown are the mean +/- SEM of triplicate determinations. These levels represent approximately 60% of added label.


3HcAMP (cpm x 10-4)
0
1
2
3
4
5
6
10
20
30
40
50
60
70
3H Adenine labeling time (mins)
3H ATP + ADP cpm x 10-5
1
2
0
20
40
60

labeling. A small but significant ($p < 0.01$, 1 x ANOVA blocked) decrease in ^{3}H-nucleotide levels was noted after 60 min of ^{3}H-adenine labeling. A 40 min preincubation time was therefore routinely adopted in an attempt to stabilize apparent basal activity, from which hormonal effects on ^{3}H-cyclic AMP levels could be assessed.

1.2. Effect of Vessel Size on Basal Activity and pH.

Stabilization of ^{3}H-cyclic AMP basal activity also required rapid mixing to ensure adequate suspension of the vesicular preparation (Fig. 4). Incubation of the bulk-labeled preparation in 1.5 ml conical tubes (10.8 mm max. diameter) resulted in inadequate suspension of the preparation such that, on mixing, basal activity rose relative to unperturbed controls (Fig. 4A). Mixing did not perturb basal activity when incubations were conducted in scintillation vials (24 mm diameter) with rapid mixing (Fig. 4B). Basal activity declined with increased ^{3}H-adenine labeling time (Fig. 4, not apparent in Fig. 3 due to scale of Y-axis). Because shaking conditions were changed, a rexamination of incubation pH conditions at various times during the assay was conducted.

The pH of 25 mM KRB slowly rose from 7.4 to 7.8-7.9 during pre- and post-labeling periods (Table 4). The pH of buffer in the presence of tissue was determined on supernatants following a 30 sec microcentrifugation. The

Fig. 4. Effect of mixing the vesicular preparation on basal ^{3}H-cyclic AMP levels in two incubation vessels.

A vesicular preparation from 2 guinea pig cerebral cortices was prepared and bulk-labeled in 25 mM KRB for 40 min, as described in Fig. 1. ^{3}H-adenine was added and bulk-labeling continued for 25 min. Aliquots (0.2 ml) were then distributed to 1.5 ml conical ependorff tubes (A) or glass scintillation vials (B), both containing 0.75 ml of buffer; the air space was flushed with 95% O_2-5% CO_2, vessels sealed and incubation continued for a further 20 min with rapid shaking (3 cycles/sec). At this time (t = 0 x-axis) either buffer (50 ul) was added and the samples gently manually mixed, flushed with 95% O_2-5% CO_2, and resealed (●·····●) or samples were not disturbed (■————■), and the subsequent time course of ^{3}H-cyclic AMP levels was determined.

Values are means +/- SEM for triplicate determinations, normalized with respect to initial basal activity for A and B separately (t = 0 x-axis). These results were replicated under similar conditions.

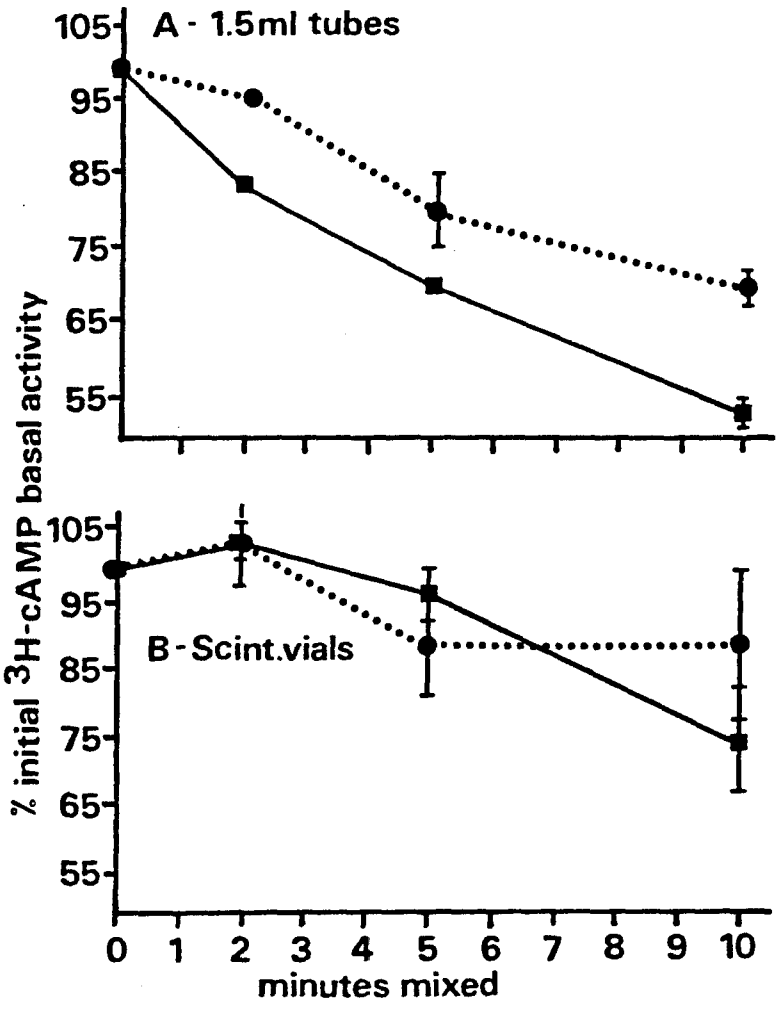

A - 1.5ml tubes
B - Scint.vials
% initial ^{3}H-cAMP basal activity
105
95
85
75
65
55
0 1 2 3 4 5 6 7 8 9 10
minutes mixed

Table 4. pH of buffers under different incubation conditions.

Incubation Condition	Preincubation	^{3}H-Adenine Labeling	Antagonist Preequilib-ration	Agonist Incubation
	(40 min)	(25 min)	(20 min)	(10 min)
25 mM KRB Bulk Labeling	7.50	7.75	7.85	8.35 (a)
Phosphate Bulk Labeling	7.25	7.35	7.45	7.45 (a)
Hepes Individual Labeling	7.35	7.35	7.45	7.45 (a)
15 mM KRB Individual Labeling	7.45	7.45	7.55	7.65

Shown are pH measurements with buffer incubations (alone) obtained at various stages of vesicular incubation (see Fig. 1 and methods for dilution steps). The numbers in parentheses indicate the duration of each step, after which the pH measurement was made. Incubation was in sealed vials under 95% O_2-5% CO_2, except where indicated (a) which was done under air. Each value represents the mean from 2 separate experiments, each determined in quadruplet.

pH of buffer or buffer plus tissue supernatants was about Ø.2 pH units higher than uncentrifuged values. Buffer pH was uncompromised by tissue (not shown). A rapid rise in extracellular pH occurred when vials were left open to air (Table 4). The observed pH changes in 25 mM KRB were presumably due to inadequate maintenance of aqueous CO_2 content. Decreasing the bicarbonate concentration to 15 mM or to 4 mM supplementing with phosphate or hepes maintained the pH at around 7.4 - 7.6, although a small time-dependent rise in pH still occurred (Table 4). These latter three buffers, i.e. 15 mM KRB, phosphate and hepes, were compared in further studies.

1.3. Variation of Basal and Histamine-Stimulated ^{3}H-Cyclic AMP Within a Given Incubation Condition.

1.3.1. Influence of ^{3}H-Adenine Labeling Time on ^{3}H-Cyclic AMP Levels.

In bulk-labeled preparations, both apparent basal (see above) and absolute HA ^{3}H-cyclic AMP levels declined as a function of ^{3}H-adenine labeling time. This phenomenon was investigated in phosphate buffer by comparing HA concentration-response curves obtained at different ^{3}H-adenine labeling times, with a randomized design for the addition of various HA concentrations (Fig. 5A). With extended ^{3}H-adenine labeling times, a significant decline in absolute HA stimulation was observed across all HA

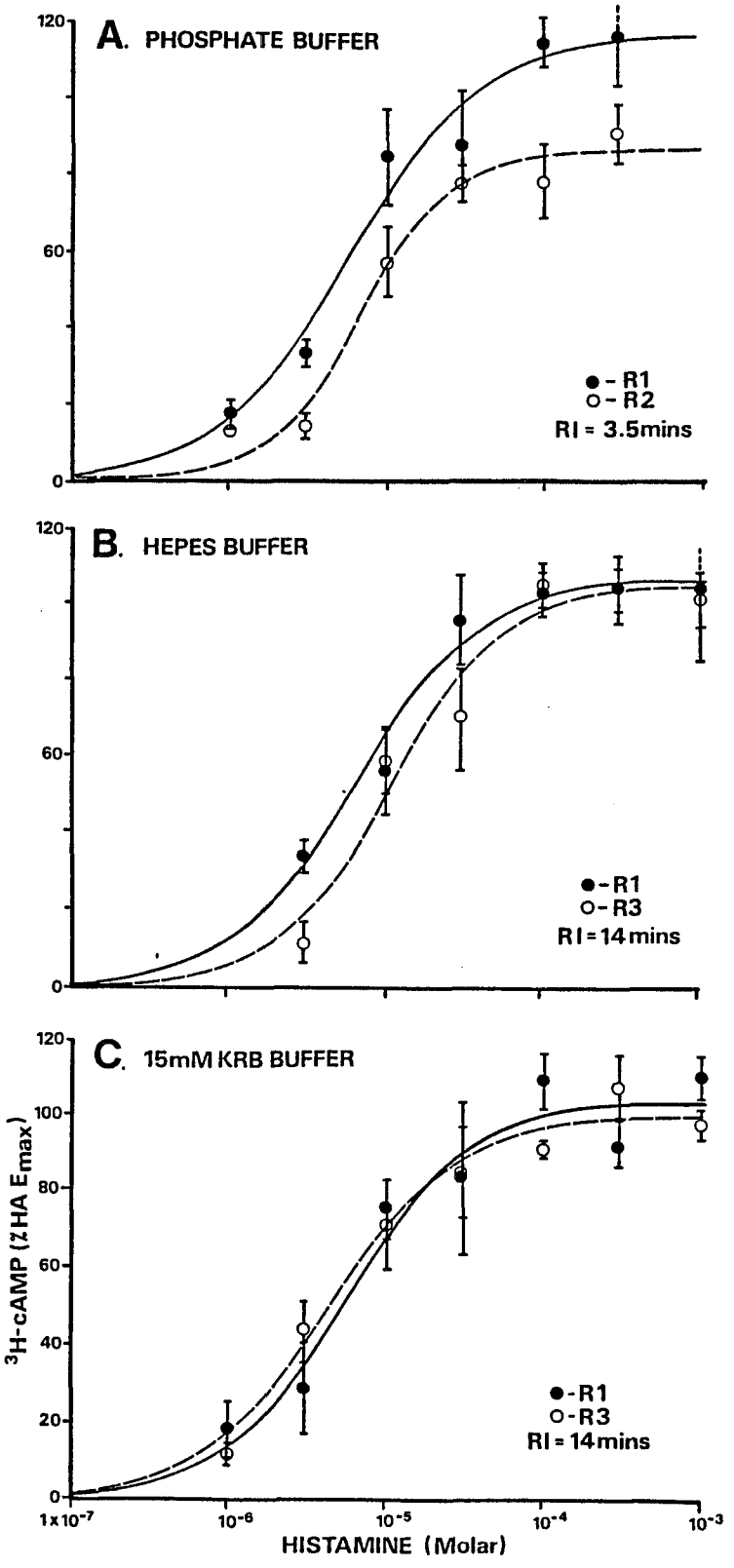
Fig. 5. Effect of incubation conditions on the stability of histamine-stimulated ^{3}H-cyclic AMP levels in the vesicular preparation.

Vesicular preparations of guinea pig cerebral cortices were prepared and incubated as described in methods, for bulk-labeling in phosphate (A), individual-labeling in hepes (B) and individual-labeling in 15mM KRB (C).

Following ^{3}H-adenine labeling, HA (Ø - Ø.3 mM) was added for 1Ø min. HA-stimulated ^{3}H-cyclic AMP accumulation in phosphate (A) was determined between 45 - 52 min after ^{3}H-adenine labeling. In hepes (B) or 15 mM KRB (C) all HA-stimulated ^{3}H-cyclic AMP values were determined after 45 min of ^{3}H-adenine labeling.

For each experiment, single determinations of all HA concentrations were obtained (R1) followed by a second (R2) and then a third replicate (R3; B and C only). For each HA concentration, the time between replicate determinations was fixed (RI).

Shown is the mean +/- SEM of 4 experiments (for each incubation condition A - C). Replicate determinations are normalized (1ØØ%) to the HA E_{max} obtained from a logistic fit to the mean of replicate determinations obtained in each experiment.


A. PHOSPHATE BUFFER
120
60
0
● - R1
○ - R2
RI = 3.5mins
B. HEPES BUFFER
120
60
0
● - R1
○ - R3
RI = 14 mins
C. 15mM KRB BUFFER
120
100
80
60
40
20
0
^{3}H-cAMP (%HA E_{max})
● - R1
○ - R3
RI = 14 mins
1x10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
HISTAMINE (Molar)

concentrations ($p < 0.001$; ANOVA 2 x blocked; n = 4). This decline was apparent as early as 3.5 min between replicates (Fig. 5A). The HA EC_{50} and slope index were not influenced by ^{3}H-adenine labeling times. Interestingly, within a given experiment, fold HA (300 uM) stimulation did not decline with increasing ^{3}H-adenine labeling time ($p > 0.05$ paired t-test n = 6). However, in several instances high concentrations of HA (300 uM) gave lower ^{3}H-cyclic AMP levels than low concentrations (10 uM) added less than 1 min earlier (not shown). This latter observation illustrates the technical difficulties inherent in constructing accurate pharmacological profiles using bulk ^{3}H-adenine labeled vesicular preparations.

Several experiments showed that bulk-labeled preparations in 25 mM KRB also exhibited a similar decline in basal and absolute HA-responsiveness (not shown). This decline was not arrested by placing the bulk-labeled preparation on ice before distribution to scintillation vials for further incubation (not shown).

I tested the hypothesis that the decline in response to HA (and basal) was a function of total exposure time to ^{3}H-adenine by individually labeling aliquots of the vesicular preparation in scintillation vials in hepes or 15 mM KRB. Thus, ^{3}H-adenine labeling time was constant. Under these conditions, ^{3}H-nucleotide formation reached an

apparent steady-state level by around 40 min, giving similar levels (cpm) to bulk-labeled preparations (see legend to Fig. 3). No significant changes in apparent ^{3}H-cyclic AMP basal activity (not shown) or in HA concentration-response curve parameters were observed over a 14 min period when incubations were conducted in either hepes (Fig. 5B) or 15 mM KRB (Fig. 5C). These observations suggest that individual-labeling in these buffers produces a more stable HA response than bulk-labeled incubations.

1.3.2. Relationship Between Basal and Histamine-Stimulated ^{3}H-Cyclic AMP Levels.

Within a given experimental group HA-responsiveness appeared related to basal ^{3}H-cyclic AMP levels (see Fig. 6 for mean values). A significant correlation between apparent basal and absolute HA-stimulated ^{3}H-cyclic AMP levels was noted in individually-labeled preparations in hepes (r^2 = 0.703, n = 12, $p < 0.01$) and 15 mM KRB (r^2 =0.446, n = 24, $p < 0.05$). In bulk-labeled preparations in phosphate buffer the correlation was not significant but borderline (r^2 = 0.552, n = 12, $p < 0.1$). Interestingly, fold HA-stimulation was inversely related to basal activity in bulk-labeled phosphate preparations (r^2 = -0.572, n = 13, $p < 0.05$) but not in individually-labeled preparations in hepes (r^2 = 0.197, n = 12, $p > 0.5$) or 15 mM KRB (r^2 = -0.325, n = 24, $p > 0.1$).

1.4. Differences in Basal and Histamine-Stimulated ^{3}H-Cyclic AMP Between Different Incubation Conditions.

Comparison of different incubation conditions also revealed significant differences in both basal and HA-responsive ^{3}H-cyclic AMP levels (Fig. 6). Basal activity following bulk-labeling in phosphate or individual labeling in 15 mM KRB was significantly higher than individual labeling in hepes ($p < 0.05$, ANOVA 1 x unblocked and NK). The maximal response to HA in individually labeled preparations was decreased compared to bulk-labeled preparations in phosphate ($p < 0.01$ absolute and fold ANOVA 1 x unblocked and NK).

Within individually labeled preparations, incubation in 15 mM KRB gave significantly higher HA-stimulated absolute ^{3}H-cyclic AMP levels ($p < 0.01$, t-test) and fold ($P < 0.05$) compared to incubation in hepes buffer. Curiously, the first 3 experiments in hepes gave a 3.5 fold stimulation but could not be replicated (not shown). The subsequent 12 experiments were consistently lower than these 3 experiments and comprise the results shown.

Differences in HA potency between preparations were also noted (Table 5). Under all incubation conditions, HA concentration-response curves appeared monophasic. Fitted HA E_{max} values were not significantly different from

Fig. 6. Effect of incubation conditions on basal and histamine-stimulated ^{3}H-cyclic AMP levels.

Vesicular preparations of guinea pig cerebral cortices were prepared and incubated as described in Methods, for bulk-labeling in phosphate, individual labeling in hepes and individual labeling in 15 mM KRB. Following 45 min of ^{3}H-adenine labeling, buffer (▣) or HA (300 uM, □) was added for 10 min.

Shown are the mean +/- SEM for (n) number of experiments, each determined in duplicate or triplicate. Fold HA stimulation is shown within absolute HA values.

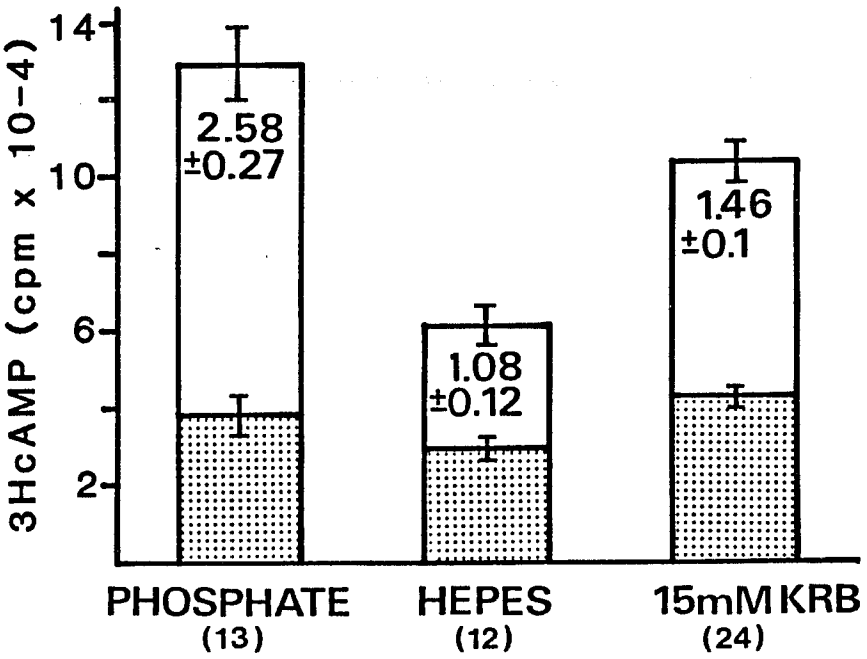

3HcAMP (cpm x 10-4)
14
10
6
2
2.58
±0.27
1.08
±0.12
1.46
±0.1
PHOSPHATE
(13)
HEPES
(12)
15mM KRB
(24)

Table 5. Effect of incubation conditions on histamine concentration-response curve parameters.

Incubation Conditions	No. Expts.	EC50 uM		Slope Index	
		mean (a)	95% C.L.	mean (b)	95% C.L.
Phosphate Bulk Labeling.	8	9.14	7.10 - 11.76	1.20	0.96 - 1.45
Hepes Individual Labeling.	10	10.88	8.39 - 14.11	1.12	0.69 - 1.55
15mM KRB Individual Labeling.	24	5.48 (c)	4.52 - 6.66	1.13	1.00 - 1.27

Vesicular preparations of guinea pig cerebral cortices were prepared, labeled and incubated as described in Methods. HA concentration-response curves were determined in duplicate (phosphate) or triplicate (hepes and 15 mM KRB) with a randomized design, as detailed in Methods. Shown are the mean +/- 95% confidence limits obtained from fitting mean HA data points from each individual experiment to a logistic function (see Methods).

(a) geometric mean.

(b) arithmetic mean.

(c) $p < 0.01$, significantly different from phosphate or hepes, 1 way unblocked ANOVA and NK.

300 uM HA-stimulated levels (see Fig. 6). Slope indices of HA concentration-response curves were not significantly different between incubation conditions ($p > 0.05$ ANOVA 1-way). HA was most potent in 15 mM KRB, as assessed by the significantly lower EC_{50} value compared to incubations in phosphate or hepes.

Since individual ^{3}H-adenine labeling in 15 mM KRB gave a stable reproducible ^{3}H-cyclic AMP response to HA, this incubation protocol was utilized in the remaining studies that have pharmacologically characterized this HA-mediated response.

2. Characterization of Histamine-Mediated ^{3}H-Cyclic AMP Accumulation.

2.1. Characterization of ^{3}H-Cyclic AMP Responses in the Absence of Adenosine Deaminase and EGTA.

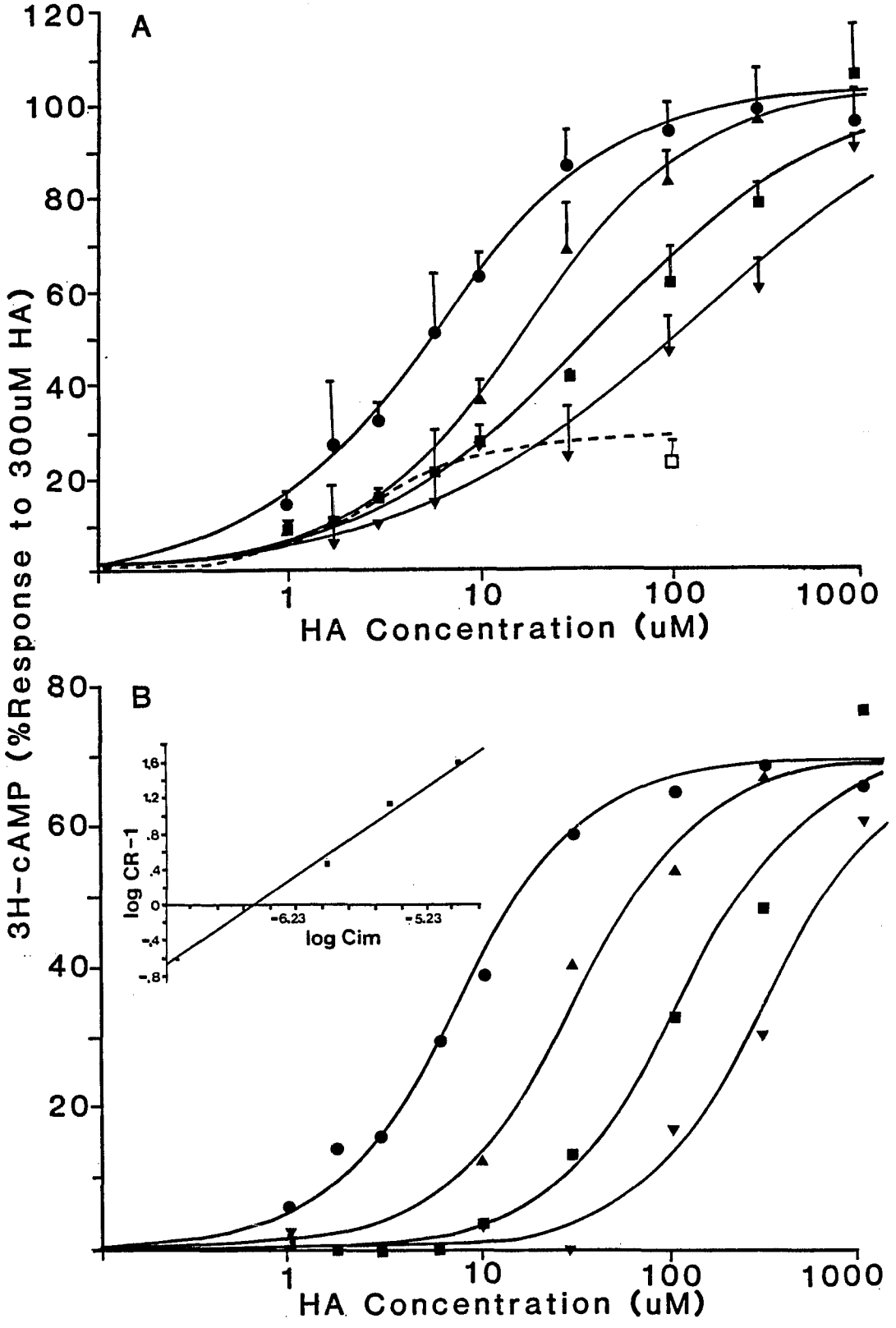
2.1.1. Effect of Histamine-Receptor Antagonists on the Histamine Response.

Cimetidine caused a concentration-dependent, surmountable shift to the right in the response to HA, at concentrations compatible with H_2-receptor antagonism (Fig. 7A). Increasing concentrations of cimetidine lowered the slope index of HA concentration-response curves (Fig. 7A; slope index = Ø.91, Ø.95, Ø.7Ø, Ø.6Ø vs Ø, 1, 3, and 1Ø uM cimetidine respectively). This significant deviation from parallelism ($F(3,24) = 3.82$; $p < Ø.Ø5$, variance ratio test on a common slope fit to data shown in Fig. 7A) suggested that, under the incubation conditions studied, the response to HA was not exclusively mediated by direct H_2-receptor stimulation.

Increasing concentrations of cimetidine revealed an apparent cimetidine-resistant component in the response to HA, accounting for approximately 3Ø% of the maximum response to HA (Fig. 7A). This component appeared dependent on HA-concentration, with an estimated $EC_{5Ø}$ of 3 uM (see Fig. 7A and legend). Subtracting this component from the overall HA response had no effect on the HA $EC_{5Ø}$

Fig.7. Cimetidine antagonism of the ^{3}H-cyclic AMP response to histamine, in the absence of adenosine deaminase.

Vesicular preparations from 2-3 guinea pig cortices were prepared and incubated in 15 mM KRB, as described in Methods. Cimetidine [Ø (●), 1 (▲), 3 (■), 1Ø (▼) and 3ØØ (□) uM] was added 2Ø min prior to incubation with HA, which was for 1Ø min. A. Each point for cimetidine inhibition is the mean +/- SEM of 5 separate experiments, (except for 3ØØ uM cimetidine which is the mean +/- SEM of 2 experiments), each determined in triplicate. Data are normalized (1ØØ%) to 3ØØ uM HA control values determined on each experiment (n = 1Ø for control). Each curve represents the logistic fit to its respective cimetidine concentration, constraining all curves to a common Emax. An apparent cimetidine-resistant component of the HA response was fit to a logistic function (by fitting to the mean values obtained by pooling all cimetidine data points from 1 - 6 uM HA) (shown as dotted line; E_{max} = 3Ø%; $EC_{5Ø}$ = 3 uM; slope index = 1.362). B. The observed increase in ^{3}H-cyclic AMP formation was corrected for the cimetidine-resistant component described in A by subtracting this function from all data points. Inset: Schild plot derived from data in B. The regression of log [CR-1] vs log [cimetidine] results in a straight line with a slope 1.11 +/- Ø.Ø9, not significantly different from 1. The pA_2 of cimetidine (6.57 +/- Ø.26) was obtained from a line where the slope is constrained to 1 (shown here).


A
120
100
80
60
40
20
1
10
100
1000
HA Concentration (uM)
3H-cAMP (%Response to 300uM HA)
B
80
60
40
20
log CR-1
1.6
1.2
.8
.4
0
-.4
-.8
-6.23
-5.23
log Cim
1
10
100
1000
HA Concentration (uM)

(6.23 vs 7.85 uM) or slope index (Ø.91 vs 1.Ø9). In 2 additional experiments, 22.1 +/- 5.6% of the response to 1ØØ uM HA was not blocked by 3ØØ uM cimetidine (Fig. 7A).

The cimetidine-sensitive component of the HA response appeared to be mediated through classical H_2-receptor stimulation. Cimetidine caused surmountable, concentration-dependent shifts of the remaining HA response, which did not deviate from parallelism ($F(3,24) = Ø.834$, $p > Ø.Ø5$ variance ratio test on common slope fit to data shown in Fig. 7B). Schild analysis of these data (Fig. 7B inset) generated a line whose slope did not differ from unity and a pA_2 for cimetidine of 6.57 +/- Ø.26.

The selective H_1-antagonist mepyramine produced concentration-dependent changes in the ^{3}H-cyclic AMP response to HA at concentrations compatible with an H_1-receptor mediated involvement in the response to HA. However, the pattern of inhibition was inconsistent with a direct independent H_1-mediated component in the HA response (Fig. 8). Mepyramine (Ø.Ø1 uM) produced a small surmountable shift to the right in the HA-concentration response curve in 2 of the 3 experiments pooled in Fig. 8 ($EC_{5Ø}$ = 6.4, 6.2 vs 1Ø.4 and 1Ø.3 uM, respectively). In the third experiment no significant shift was noted ($EC_{5Ø}$ = 6.55 vs 6.85 uM). This small shift to the right in the HA response is about 1Ø fold less than that predicted for

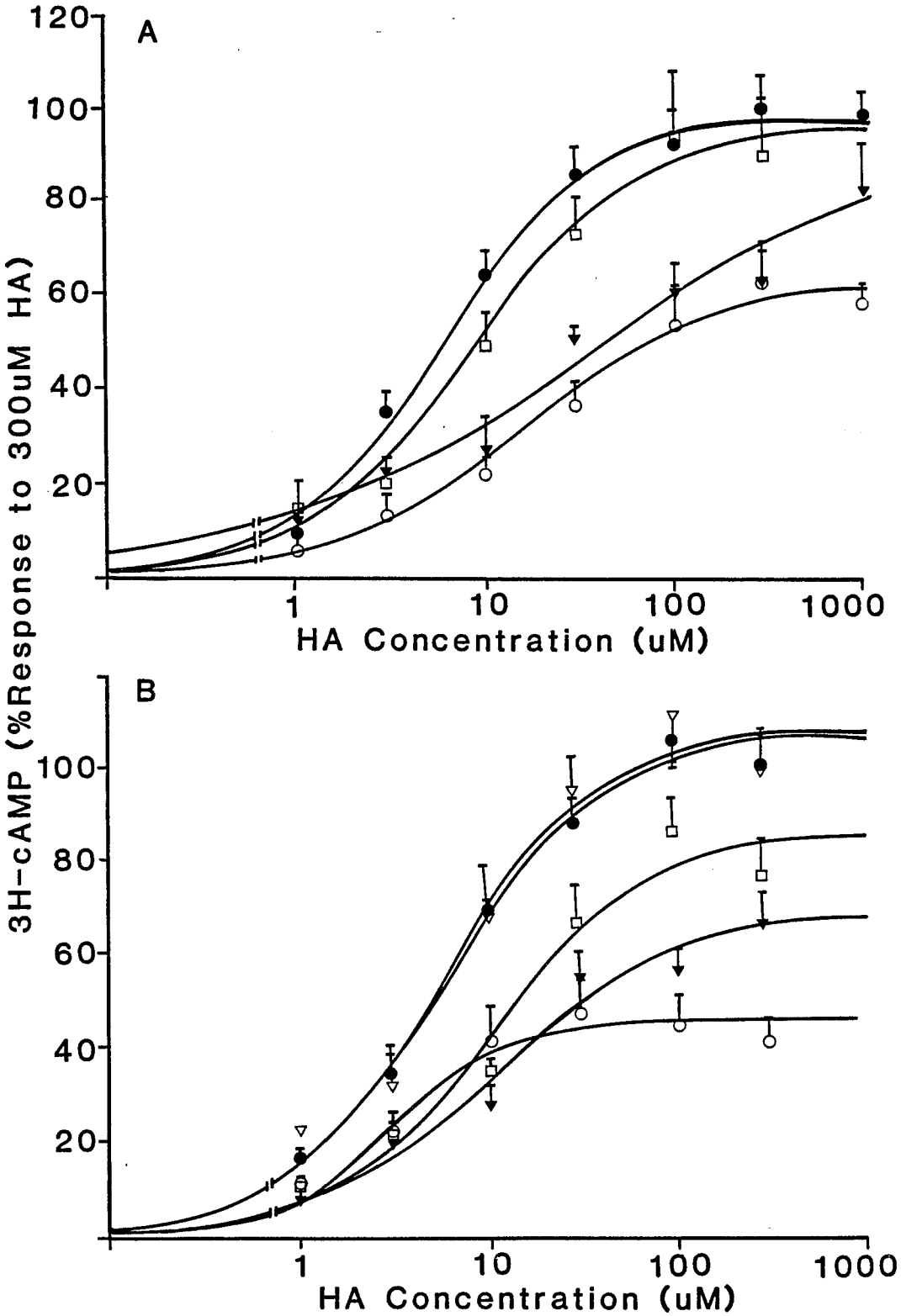
Fig. 8. Effect of H_1-antagonists on the histamine concentration-response curve, in the absence of adenosine deaminase.

Vesicular preparations were prepared from 2 guinea pig cortices and incubated in 15 mM KRB, as described in Methods. The H_1-antagonists mepyramine (A) and chlorpheniramine (B) were added 20 min prior to incubation with HA, which was for 10 min.

A. Mepyramine [0 (●), 0.01 (□), 0.1 (▼) and 1.0 (○) uM; n = 6, 3, 3, and 3 respectively].

B. d-chlorpheniramine [0 (●), 0.01 (□), 0.1 (▼) and 1.0 (○) uM; n = 4, 2, 3, and 2 respectively].
l-chlorpheniramine [0.1 uM (▽) n = 2].

Each point represents the mean +/- SEM of n separate experiments, each determined in triplicate. Data are normalized (100%) to 300 uM HA control values determined in each experiment. Curves shown are logistic fits to the mean data points.


A
120
100
80
60
40
20
3H-cAMP (%Response to 300uM HA)
1
10
100
1000
HA Concentration (uM)
B
100
80
60
40
20
1
10
100
1000
HA Concentration (uM)

classical competitive H_1-receptor antagonism alone.

Increasing concentrations of mepyramine caused a further reduction in the HA response, but with no further shift in the overall HA EC_{50} (Fig. 8). While 0.1 and 1.0 uM mepyramine consistently inhibited the response to 300 uM HA, increasing the HA concentration to 1 mM overcame the inhibition to 0.1 uM mepyramine. In one additional experiment, 0.1 M HA overcame the inhibition to 1.0 uM mepyramine, although lower HA concentrations were inhibited to a similar extent (not shown, similar pattern to Fig. 8A). The effects of 0.1 and 1.0 uM mepyramine on the HA response were similar, suggesting that, at these concentrations, the mepyramine-sensitive component of the HA response was approaching maximal inhibition i.e. only a fraction of the HA response was sensitive to inhibition by mepyramine.

The hypothesis that mepyramine exerts its effects through an action at H_1-receptors was supported by experiments with the H_1-antagonists d- and l-chlorpheniramine. The pattern of inhibition of the HA response with d-chlorpheniramine was similar to that noted for mepyramine (Fig. 8A vs 8B), i.e. all concentrations of d-chlorpheniramine caused a small shift to the right in the HA EC_{50} and increasing concentrations caused a depression in the maximal HA response.

Importantly, the stereoisomer l-chlorpheniramine (Ø.1 uM), which has about 1ØØ times less affinity for H_1-receptor binding sites than d-chlorpheniramine (Table 1), had no significant effect on the HA-response (Fig. 8B). In one experiment (not shown) increasing concentrations of l-chlorpheniramine to 1 and 1Ø uM caused a pattern of inhibition similar to those noted for Ø.1 and 1 uM d-chlorpheniramine.

Only 45% of the ^{3}H-cyclic AMP response to HA (1ØØuM) was sensitive to inhibition by H_1-antagonists (Fig. 9). A logistic fit to all the data of Fig. 9 (see legend Fig. 9) yielded IC5Ø values of 36 +/- 8, 14 +/- 3 and 767 +/- 17Ø nM for mepyramine, d-chlorpheniramine and l-chlorpheniramine respectively, and generated a value of 54.67 +/- 1.1% for the H_1-antagonist insensitive component. These values were not significantly altered when each antagonist curve was fit individually. Maximal inhibition was attained by 1.Ø uM mepyramine and d-chlorpheniramine, with no further inhibition noted when antagonist concentrations were increased by an order of magnitude. Similarly, l-chlorpheniramine maximally inhibited the HA-response at about 1Ø uM, with no further inhibition noted at 1ØØ uM.

The maximal inhibition by H_1-antagonists of the HA response (45%) was about 5Ø% larger than the cimetidine-resistant component (22-3Ø%) described above (cf. Fig. 7

Fig. 9. Effect of H_1-antagonists on the ^3H-cyclic AMP response to 100 uM histamine, in the absence of adenosine deaminase.

Vesicular preparations from 2-3 guinea pigs were prepared and incubated in 15 mM KRB, as described in Methods. Mepyramine (■), d-chlorpheniramine (▽) and l-chlorpheniramine (▼) were added 20 min prior to incubation with HA (100 uM), which was for 10 min.

Each point represents the mean +/- SEM of 3-7 experiments, each determined in triplicate. Data are normalized to 100 uM HA control values determined in each experiment. Curves shown were generated by fitting the data to the function:

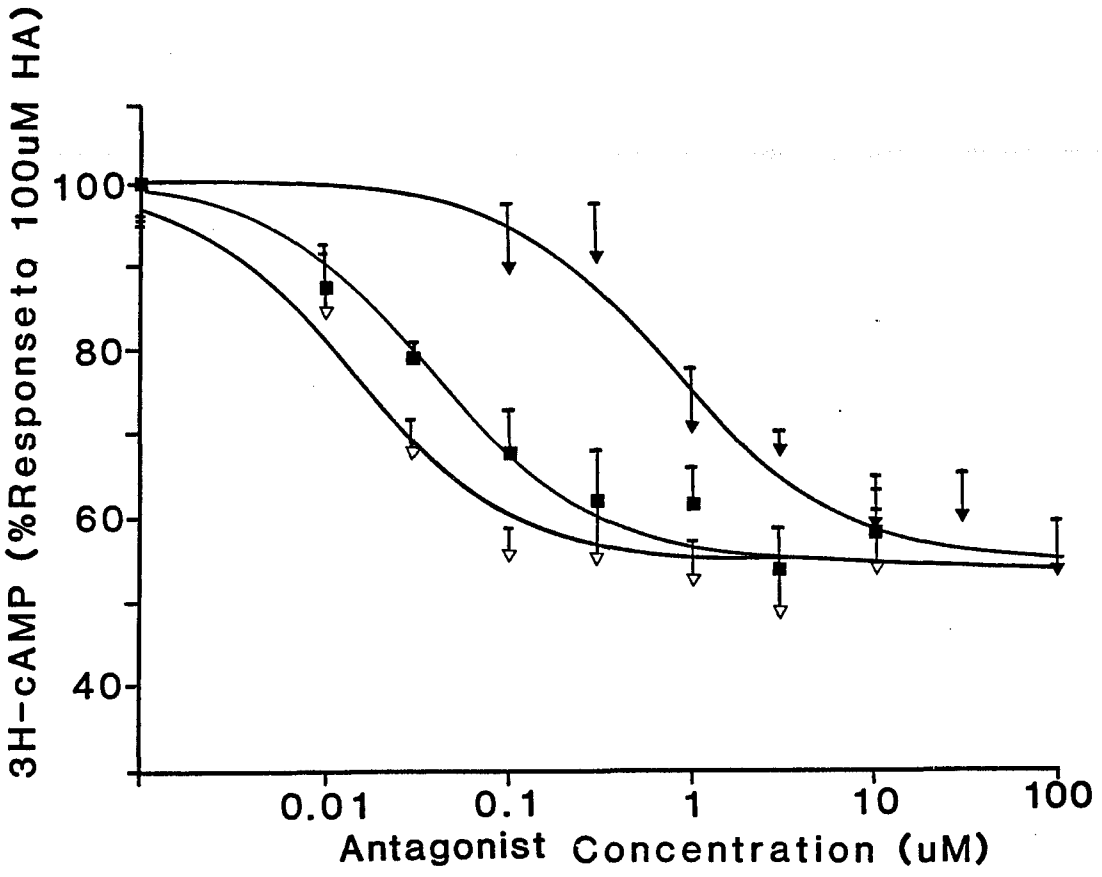
$$\text{Response} = 100 - \frac{(100 - C)}{1 + (IC_{50}/[A])}$$

where:

C = fractional percent of the HA response insensitive to H_1-antagonists.

[A] = concentration of antagonist.

IC_{50} = [A] producing half maximal effect.


3H-cAMP (%Responseto 100uM HA)
100
80
60
40
0.01
0.1
1
10
100
Antagonist Concentration (uM)

vs Fig. 9). This observation suggests that a significant proportion of the cimetidine-sensitive component of the HA-response is also inhibited by H_1-antagonists.

Higher concentrations of H_1-antagonists may cause a further inhibition in the response to HA. Increasing the concentration of mepyramine or d-chlorpheniramine to 30 uM caused a further decline in the HA response (37.5 +/- 3.4 (n = 2) and 43.2 +/- 6.0% (n = 1) of the 100 uM HA response remaining, respectively). Similarly, in one experiment, l-chlorpheniramine at 1 mM reduced the HA response to 43.2 +/- 6.0%. The nature of this inhibition was not investigated further.

2.1.2. Effect of Histamine-Receptor Antagonists on Dimaprit-Induced ^{3}H-Cyclic AMP Accumulation.

Dimaprit, the highly selective H_2-agonist, stimulated ^{3}H-cyclic AMP accumulation in a concentration-dependent manner (Fig. 10). Dimaprit concentration-response parameters derived from a logistic fit to the data of Fig. 10 (normalized to the response to 300 uM HA) were EC_{50} = 76.5 uM; slope index = 0.502; E_{max} = 68%. The shape and concentration-response parameters of the dimaprit response were similar when absolute (cpm) dimaprit stimulation was assessed (n = 9; EC_{50} = 14 uM; slope index = 0.591; E_{max} = 60% HA E_{max}) (not shown). Over the concentration range studied, the maximum response to dimaprit was

clearly less than the response to HA (Fig. 10).

In paired experiments, incubation with cimetidine (3 uM) inhibited the response to both HA and dimaprit (Fig. 11A and B). As previously noted for HA, cimetidine caused a surmountable shift in the concentration-response to dimaprit. Cimetidine antagonism of this response did not deviate from parallelism ($F(1,7) = 0.05$; common slope = 0.581, E_{max} = 56.1%). The cimetidine K_{iapp} value derived from this common slope fit, 0.49 uM (an approximation only given the low stimulation and slope index seen in these experiments), was similar to that derived for the H_2-mediated component of the HA response (0.27 uM, see Fig. 7). In contrast, a paired experimental design revealed, that while the response to HA was clearly inhibited by mepyramine (0.1 uM) this concentration of antagonist had no effect on the dimaprit concentration-response curve (Fig. 12). The response to dimaprit therefore appeared to be predominantly, if not exclusively, mediated by H_2-receptor stimulation.

Fig. 10. ^{3}H-cyclic AMP concentration-response curves to histamine and dimaprit, in the absence of adenosine deaminase.

Vesicular preparations were prepared from 2 guinea pig cortices and incubated in 15 mM KRB, as described in Methods. Final incubation (1.0 ml) with HA (●) or dimaprit (▲) was for 10 min.

Each point represents the mean +/- SEM of 9 experiments, each determined in triplicate. Data are normalized (100%) to the stimulation obtained with 300 uM HA in each experiment.

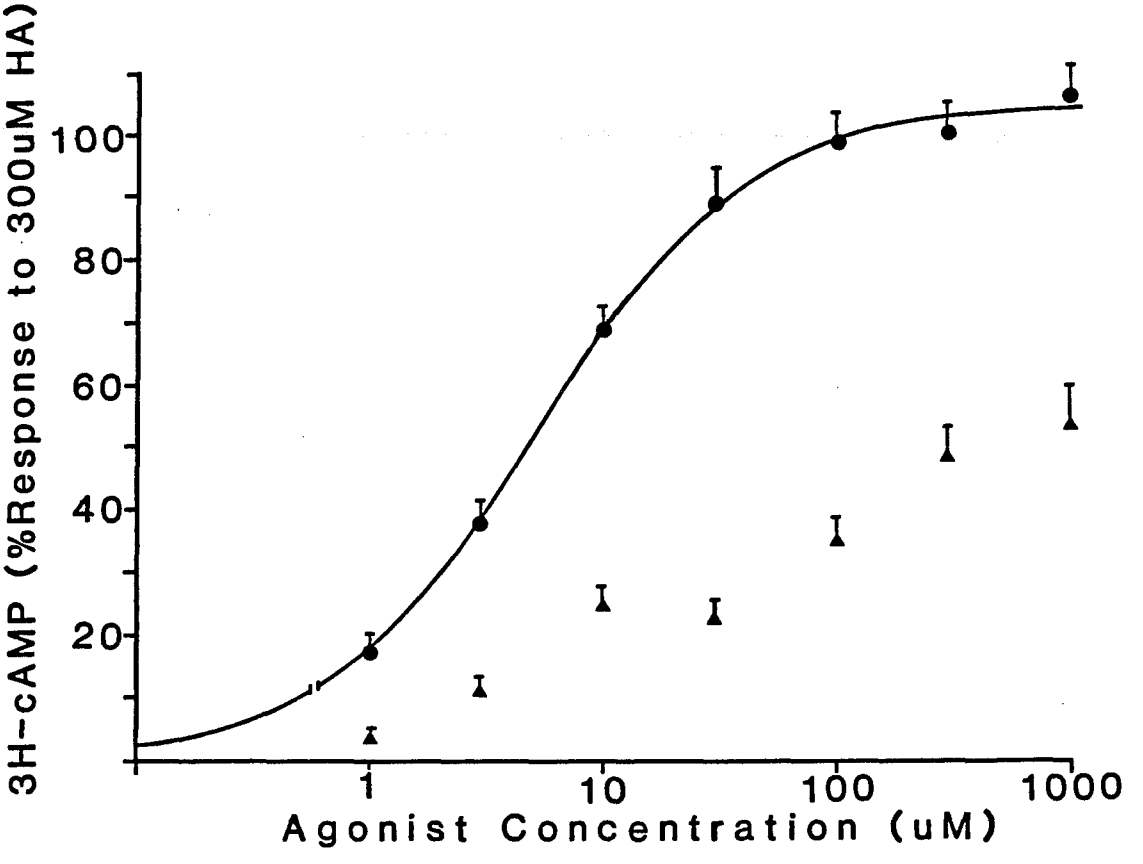

3H-cAMP (%Response to 300uM HA)
100
80
60
40
20
1
10
100
1000
Agonist Concentration (uM)

Fig. 11. Comparison of the effect of cimetidine on the ^{3}H-cyclic AMP response to histamine and dimaprit, in the absence of adenosine deaminase.

Vesicular preparations were prepared from 2 guinea pig cerebral cortices and incubated in 15 mM KRB, as described in Methods. Cimetidine (Ø (●, ▲) and 3 (○, △) uM) was preequilibrated for 20 min prior to incubation with HA (top) or dimaprit (bottom), which was for 10 min.

Each point represents the mean +/- SEM of 9 data points, from 3 separate experiments, each determined in triplicate. Data are normalized (100%) to 300 uM HA values determined in each experiment.

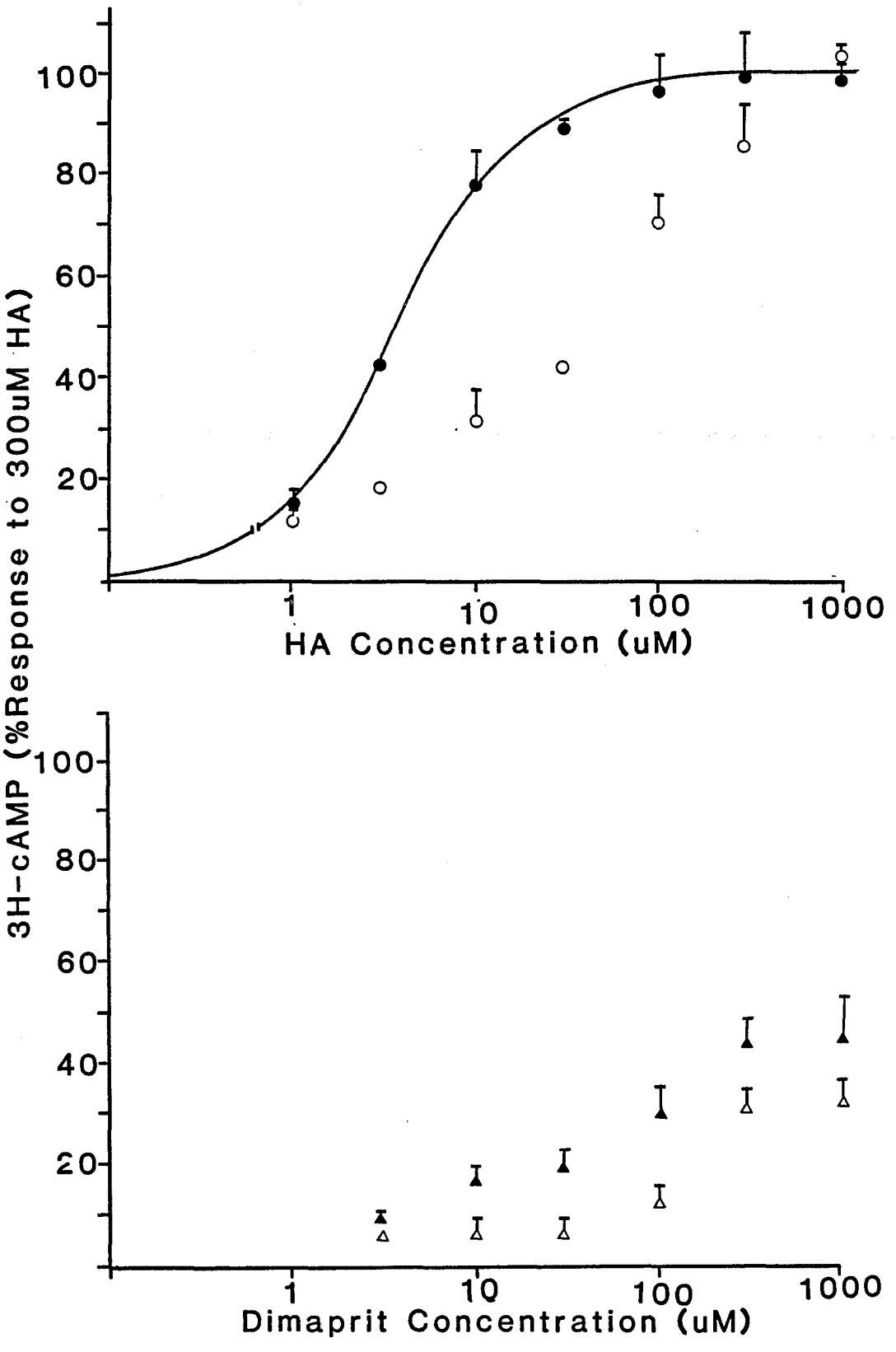
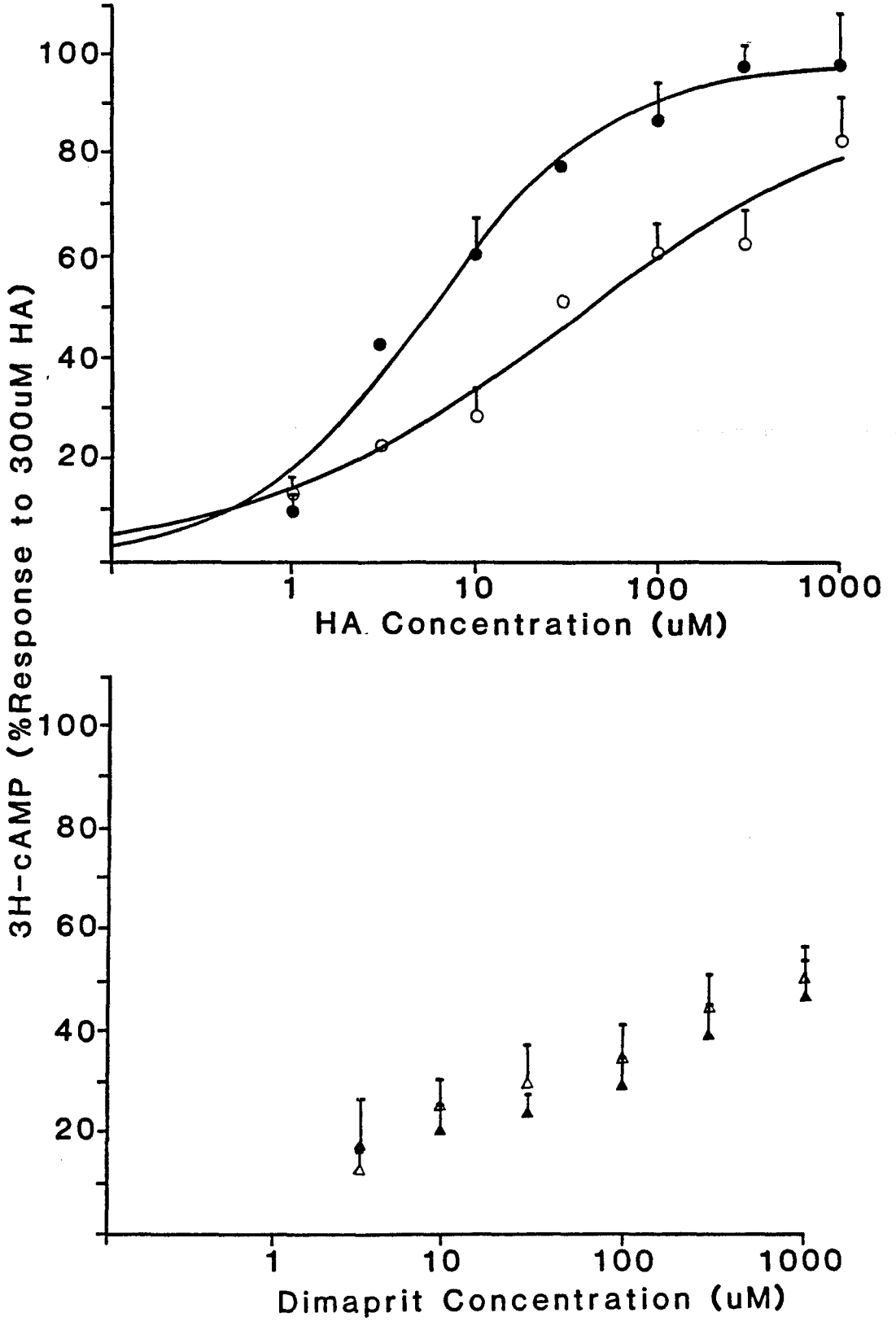

3H-cAMP (%Response to 300uM HA)
100
80
60
40
20
1
10
100
1000
HA Concentration (uM)
100
80
60
40
20
1
10
100
1000
Dimaprit Concentration (uM)

Fig. 12. Comparison of the effect of mepyramine on the ^{3}H-cyclic AMP response to histamine and dimaprit, in the absence of adenosine deaminase.

Vesicular preparations from 2 guinea pig cortices were prepared and incubated in 15 mM KRB, as described in Methods. Mepyramine [Ø (●,▲) and Ø.1 (○,△) uM] was prequilibrated for 2Ø min prior to incubation with HA (top) or dimaprit (bottom), which was for 1Ø min.

Each point represents the mean +/- SEM of 9 data points from 3 separate experiments, each determined in triplicate. Data are normalized (1ØØ%) to 3ØØ uM HA values determined in each experiment.


100
80
60
40
20
3H-cAMP (%Response to 300uM HA)
1
10
100
1000
HA Concentration (uM)
100
80
60
40
20
1
10
100
1000
Dimaprit Concentration (uM)

2.2. Characterization of Histamine-Mediated ^{3}H-Cyclic AMP Accumulation in the Presence of Adenosine Deaminase.

2.2.1. Histamine Concentration-Response Curve.

Addition of adenosine deaminase caused an 80% reduction in apparent ^{3}H-cyclic AMP basal activity (Table 6). This treatment had no significant effect on the absolute response to HA (E_{max} in Table 6). Due to the marked reduction in basal activity, fold HA stimulation was increased by a factor of about 4 (Table 6). HA concentration-response curves appeared monophasic in both the presence and absence of adenosine deaminase and the slope indices of these curves were not significantly different from 1 (Table 6). Adenosine deaminase caused a significant shift to the right in the HA EC_{50} (Table 6) indicating that this treatment changed the efficiency and/or nature of HA-receptors coupled to ^{3}H-cyclic AMP accumulation in this preparation.

2.2.2. Effect of Histamine-Receptor Antagonists on the Histamine Response.

In paired experiments, adenosine deaminase decreased both basal (Table 6) and absolute HA-stimulation (100 uM) (Fig. 13). Inclusion of adenosine deaminase also caused significant changes in the sensitivity of the HA response to inhibition by mepyramine (3 uM) and cimetidine (300

Table 6. Effect of adenosine deaminase with or without EGTA on HA concentration-response curves.

Treatment (no. expts)	Basal (cpm)	Emax (total -basal)	Fold (Emax/ basal)	EC50+ uM	Slope index
Control (28)	4544 ±252	6150 ± 423	1.41 ±0.10	5.91 4.86-7.20	1.11 0.99-1.22
2.5 U/ml adenosine deaminase (7)	943* ±51	5097 ± 449	5.45* ±0.49	11.36# 7.97-16.18	1.05 0.84-1.27
2mM EGTA + 2.5U/ml adenosine deaminase (12)	1062* ±45	2807** ± 129	2.68** ±0.14	16.22* 12.64-20.80	0.99 0.83-1.14

Vesicular preparations of guinea pig cerebral cortex were prepared and incubated in 15 mM KRB, with or without adenosine deaminase (2.5 U/ml) or EGTA (2 mM), as described in Methods. HA concentration-response curves (1-1000 uM) were determined in triplicate. Shown are the mean with either SEM (+/-) or 95% confidence limits (dashes) obtained from fitting mean HA data points from individual experiments to a logistic function (see Methods for further details). Other than the slope index, unblocked 1x ANOVA revealed significant differences between treatment groups ($p<0.001$ for all parameters). Significance levels given were determined by Newman-Keuls multiple range testing. + Geometric mean, all other values are arithmetic means. # $p<0.05$, * $p<0.01$ significantly different from control. ** $p<0.01$ significantly different from control and adenosine deaminase alone.

Fig. 13. Effect of adenosine deaminase on the sensitivity of the ^{3}H-cyclic AMP response to histamine to inhibition by cimetidine and mepyramine.

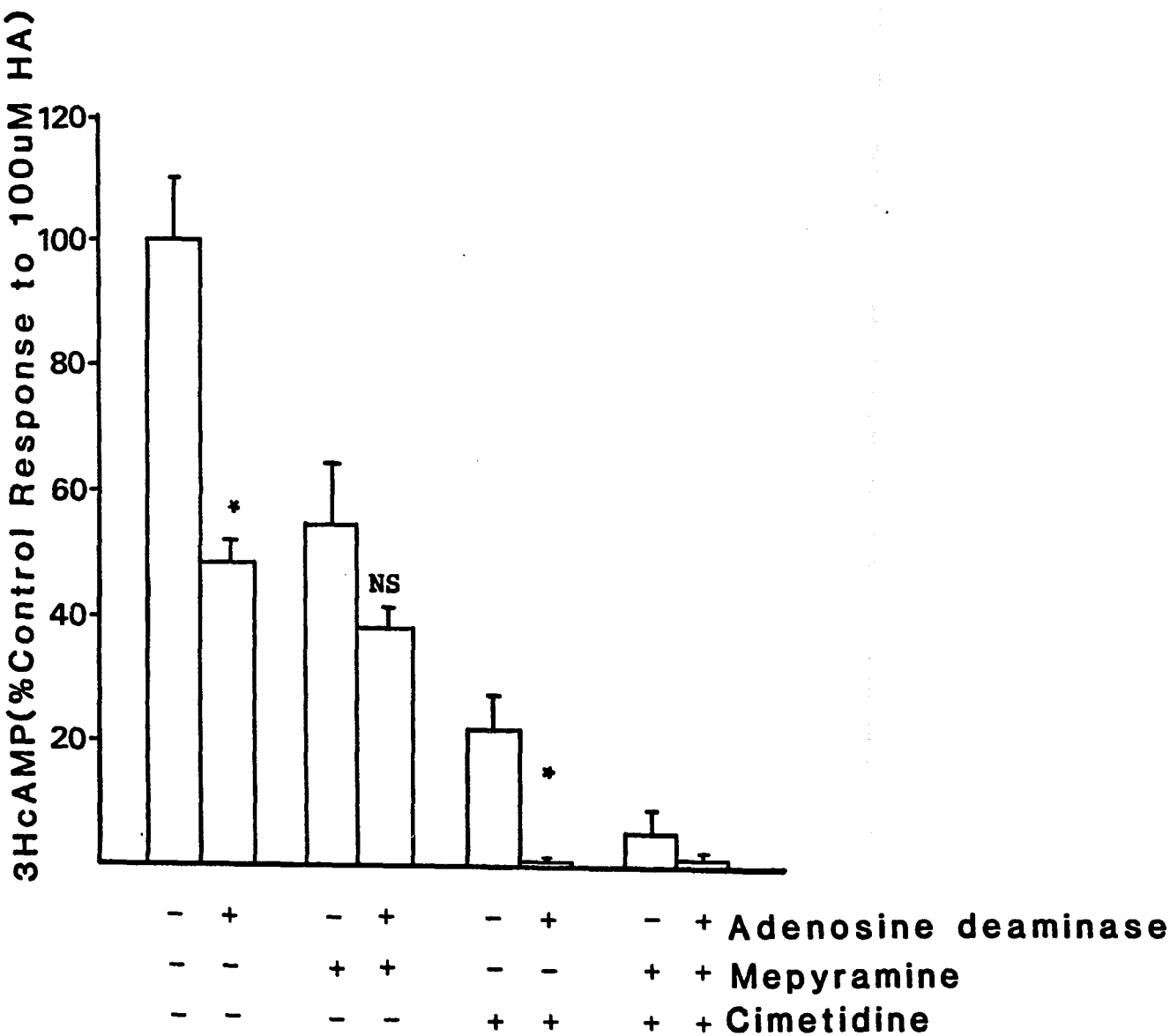
Vesicular preparations were prepared from 2 guinea pig cortices in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the absence or presence of adenosine deaminase (2.5 U/ml). Mepyramine (3.0 uM), cimetidine (300 uM) or buffer were added 20 min prior to incubation with HA (100 uM), which was for 10 min.

Shown is the mean +/- SEM of 6 data points, determined from 2 separate experiments, each in triplicate. Data are normalized (100%) to the control HA response determined in each experiment.

* $p < 0.05$, significantly different from each paired incubation in the absence of adenosine deaminase;
(n = 6; paired t-test for each treatment).

NS $p > 0.05$, not significantly different from incubation under the same conditions but in the absence of antagonist;
(n = 6, paired t-test).
All other incubations with mepyramine and cimetidine caused a significant inhibition of the corresponding HA response;
($p < 0.05$, n = 6, paired t-test).

See text for further details.


3HcAMP(%Control Response to 100uM HA)
120
100
80
60
40
20
*
NS
*
- + - + - + - + Adenosine deaminase
- - + + - - + + Mepyramine
- - - - + + + + Cimetidine

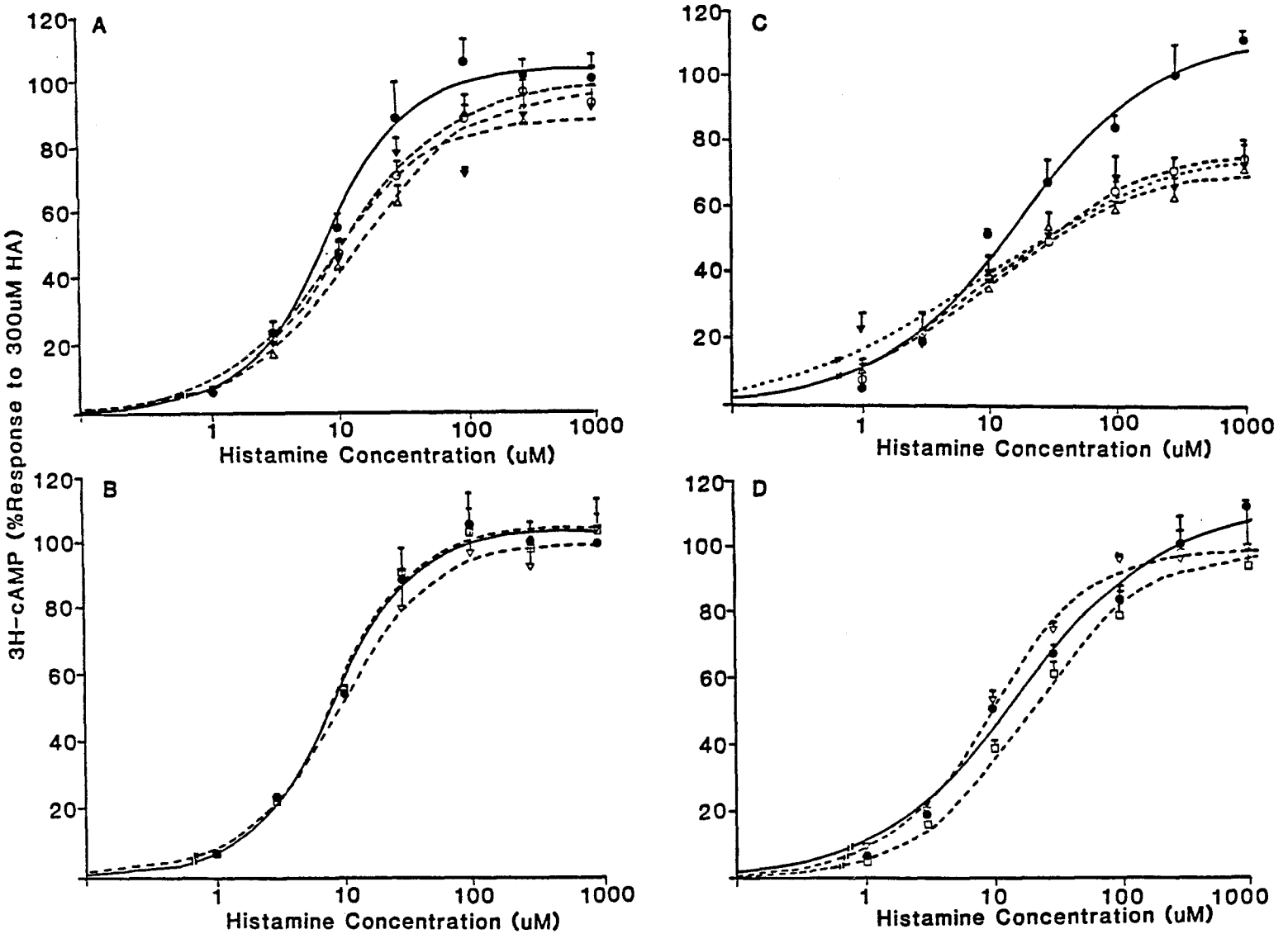
uM), compared to incubation in its absence (Fig. 13). In contrast to the absence of adenosine deaminase, in its presence the entire response to HA (100 uM) was blocked by cimetidine (300 uM), suggesting that adenosine deaminase abolished the cimetidine-resistant component noted in this study (Fig. 13) and described previously (Fig. 7). Addition of adenosine deaminase also appeared to reduce the sensitivity of the HA response to inhibition by maximally effective concentrations of mepyramine (3 uM) (Fig. 13 and see below). A combination of mepyramine (3 uM) and cimetidine (300 uM) abolished the HA response in both the presence and absence of adenosine deaminase (Fig. 13), and had no effect on basal ^{3}H-cyclic AMP basal levels (not shown). These paired studies also revealed that adenosine deaminase caused a 10% increase in ^{3}H-nucleotide levels compared to incubation in its absence (not shown).

As noted above, adenosine deaminase appeared to reduce, but not eliminate, the inhibition of the HA response by H_1-antagonists (cf. Fig. 8 vs Figs. 14A and C). In the presence of adenosine deaminase, the H_1-antagonists mepyramine, d-chlorpheniramine and promethazine (0.1 and 1.0 uM) caused similar concentration-dependent effects on the HA response (Fig. 14A and C). H_1-antagonists did not cause a shift in the HA EC_{50} when adenosine deaminase was present, as they did

Fig. 14. Effect of H_1-antagonists on the ^3H-cyclic AMP concentration-response to histamine, in the presence of adenosine deaminase.

Vesicular preparations were prepared from 3 guinea pig cortices in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the presence of adenosine deaminase (2.5 U/ml). Mepyramine (○), d-chlorpheniramine (▼), and promethazine (△) (panel A and C); or l-chlorpheniramine (▽) and diphenhydramine (□) (panel B and D); were added 20 min prior to incubation with HA (control (●)), which was for 10 min. Antagonist concentrations were 0.1 uM in panels A and B and 1.0 uM in panels C and D.

Each point represents the mean +/- SEM of 6 data points determined from 2 separate experiments, each in triplicate. Data are normalized (100%) to the response obtained with 300 uM HA in each experiment. The control curve and data are the same in panel A vs B and C vs D. Error bars for the control HA response are shown in panels A and C and have been omitted from panels B and D. Curves shown are logistic fits to the mean data points shown (see methods for further details).


A
B
C
D
3H-cAMP (%Response to 300uM HA)
Histamine Concentration (uM)
120
100
80
60
40
20
1
10
100
1000

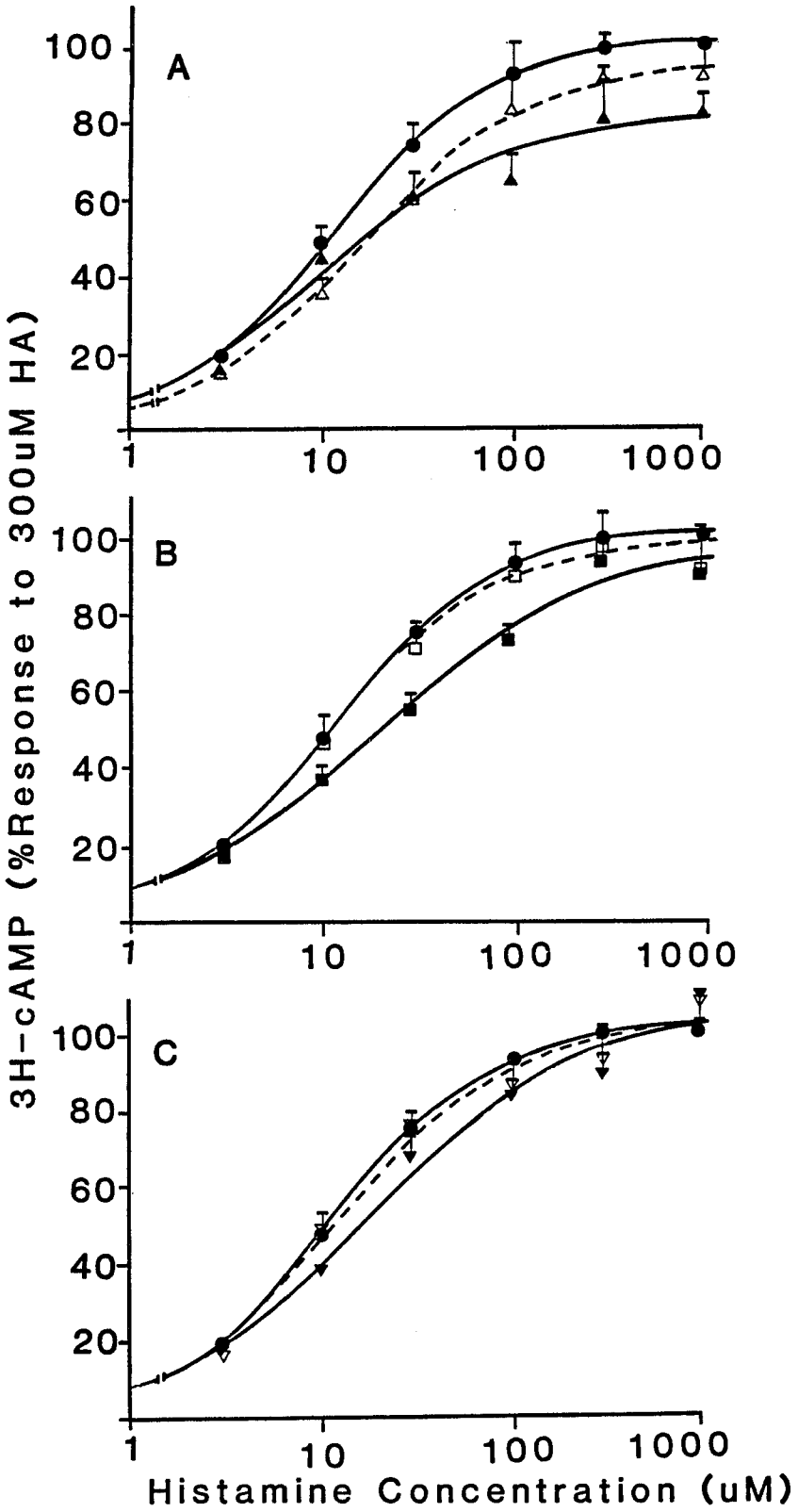
when it was absent (cf. Fig. 8 vs Fig. 14A and C). In the presence of adenosine deaminase, mepyramine, promethazine and d-chlorpheniramine (1.Ø uM) inhibited the HA E_{max} by about 30%. d-Chlorpheniramine (Ø.1 and 1.Ø uM) caused a significantly larger inhibition of the HA response in the absence than in the presence of adenosine deaminase ($p < Ø.Ø5$ control vs adenosine deaminase for each d-chlorpheniramine concentration at 1ØØ uM HA; $n > 2$; t-test). Adenosine deaminase caused a similar trend on the inhibition of the response to HA by mepyramine. Similarly, l-chlorpheniramine, at concentrations up to 1.Ø uM, had no effect on the HA response in the presence of adenosine deaminase (Fig.14 B and D) while in the absence of adenosine deaminase 1.Ø uM of this H_1-antagonist inhibited the HA response (cf. Fig.9). In the presence of adenosine deaminase diphenhydramine (Ø.1 and 1.Ø uM), an H_1-antagonist with about 1Ø times less affinity for the H_1-receptor than mepyramine, also had no effect on the HA response.

In the presence of adenosine deaminase, experiments with the tricyclic antidepressants doxepin and amitryptyline failed to demonstrate the H_1-antagonist properties of these componds. Thus, at concentrations of around 1ØØ and 1ØØØ times their affinities at known H_1-receptors, these agents had little or no effect on the ^{3}H-cyclic AMP response to HA (Fig. 15B and C). In the

Fig. 15. Effect of tricyclic antidepressants on the ^{3}H-cyclic AMP response to histamine, in the presence of adenosine deaminase.

Vesicular preparations were prepared from 3 guinea pig cortices in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the presence of adenosine deaminase (2.5 U/ml). Mepyramine [panel A; Ø.Ø1 (△) and Ø.1 (▲) uM]; doxepin [panel B; 1 (□) and 1Ø (■) nM] and amitryptyline [panel C; Ø.Ø1 (▽) and Ø.1 (▼) uM] were added 2Ø min prior to incubation with HA [control (●)], which was for 1Ø min.

Each point represents the mean +/- SEM of 6 data points determined from 2 separate experiments, each in triplicate. Both experiments gave similar results and all concentrations of each compound were determined in each experiment. Data are normalized (1ØØ%) to the response obtained with 3ØØ uM HA in each experiment. The control curve and data are the same in panels A, B and C. Error bars for the control HA response are shown in panel A only. Curves shown are logistic fits to the mean data points shown (see Methods for further details).


A
B
C
100
80
60
40
20
1
10
100
1000
3H-cAMP (%Resonse to 300uM HA)
Histamine Concentration (uM)

same experiments, 0.1 uM mepyramine (approximately 100 x K_b) clearly caused a significant effect, while 0.01 uM mepyramine had little activity (Fig. 15A).

The hypothesis that the lack of effect of doxepin and amitryptyline was due to a high degree of nonspecific binding to protein by these agents was tested in one experiment (not shown). Sequentially decreasing the protein concentration from 2.0 to 0.5 mg/ml (by dilution after ^{3}H-adenine labeling maintaining the final incubation volume at 1.0 ml, see Fig. 1 methods) did not reveal the H_1-antagonist properties of doxepin. HA (100 uM) and basal activity were linearly related to protein concentration ($r = 0.994$, $n = 4$, $p < 0.006$; and $r = 0.999$, $n = 4$, $p < 0.0005$, respectively). Mepyramine (1.0 uM) inhibition of the HA response (about 30%) was independent of the protein concentration. A lower concentration of mepyramine (0.01 uM) or doxepin (1 nM) had no significant effect on the HA response at any protein concentration tested.

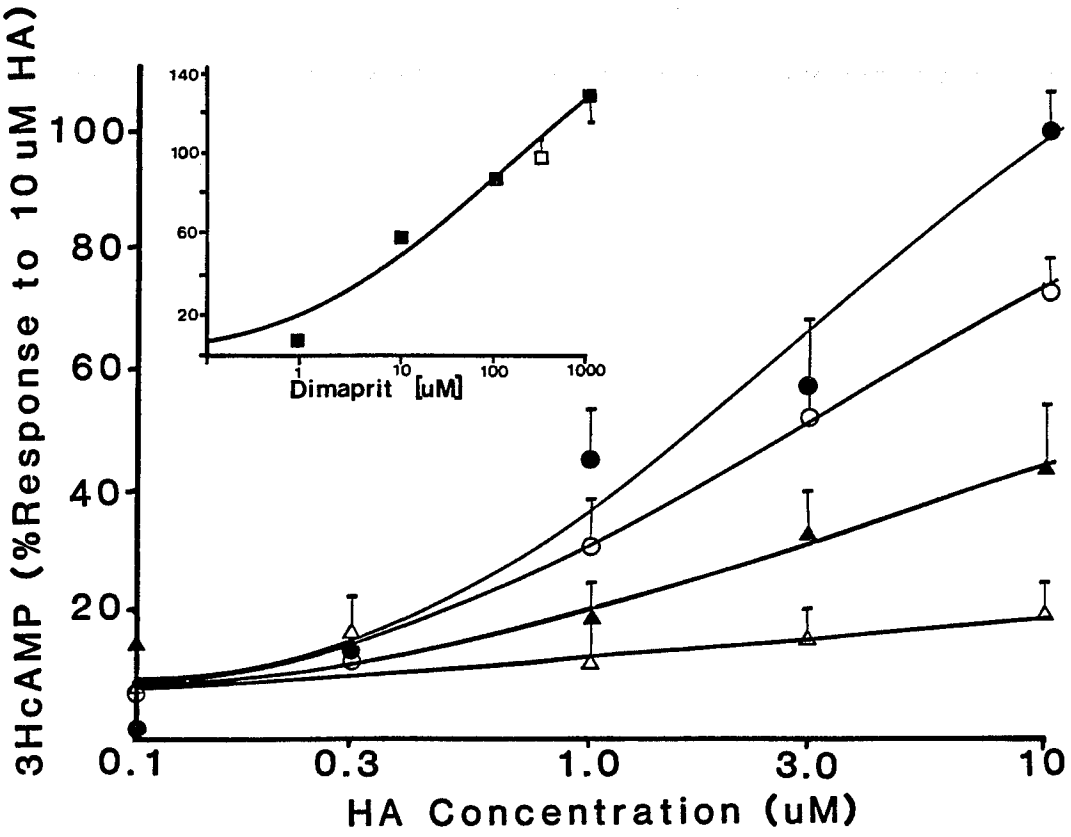
Further support for an H_1-receptor involvement in the response to HA was obtained by occupying H_2-receptors with dimaprit (100 uM). In the presence of dimaprit (100 uM) the response to HA was abolished by mepyramine (0.1uM) (Fig. 16). Adenosine deaminase was included in this study to preclude putative H_1-mediated effects due to endogenous adenosine. In 2 of the 3 experiments summarized in

Fig. 16, HA caused no significant potentiation in the response to dimaprit at concentrations less than 3 uM. Above 1 uM, the response to HA was significantly higher than the response to dimaprit alone and antagonized by Ø.1 uM mepyramine. In the presence of adenosine deaminase, as seen in its absence (Fig. 1Ø), the concentration-response to dimaprit was shallow (Fig. 16, insert; $EC_{5Ø}$ = 53.5 uM, slope index = Ø.494). The increase in ^{3}H-cyclic AMP accumulation at 1Ø uM HA might therefore reflect both H_1-potentiation of the dimaprit response and direct H_2-receptor stimulation.

Fig. 16. Effect of dimaprit on the response to histamine and its sensitivity to inhibition with mepyramine, in the presence of adenosine deaminase.

Vesicular preparations from 2 guinea pig cerebral cortices were prepared and incubated in 15 mM KRB, as described in Methods. Individual aliquots were incubated at 37 C in the presence of adenosine deaminase (2.5 U/ml). Mepyramine (Ø.1 uM) was added 2Ø min prior to incubation with HA alone or with dimaprit (1ØØ uM), which was for 1Ø min. HA (●), HA + mepyramine (○), HA + dimaprit (▲), HA + dimaprit + mepyramine (△).

Shown is the mean +/- SEM of the HA-response, obtained from 9 data points determined from 3 separate experiments, each in triplicate. The response to dimaprit alone was subtracted (▲ , △). Data are normalized (1ØØ%) to the control response obtained with 1Ø uM HA in each experiment. Curves shown were drawn by hand.
Inset: Concentration-response to dimaprit in the presence of adenosine deaminase. Each point represents the mean +/- SEM of 9 data points determined from 3 separate experiments (■), or 6 data points determined from 2 separate experiments (□), each in triplicate. Data were obtained in the same experiments as those given above and are also normalized (1ØØ%) to the control response obtained with 1Ø uM HA in each experiment. Curve is the logistic fit to the mean data points shown.


3HcAMP (%Response to 10 uM HA)
100
80
60
40
20
0.1
0.3
1.0
3.0
10
HA Concentration (uM)
140
100
60
20
1
10
100
1000
Dimaprit [uM]

2.3. Characterization of Histamine-Mediated ^{3}H-Cyclic AMP Accumulation in the Presence of Adenosine Deaminase and EGTA.

2.3.1. Histamine Concentration-Response Curve.

Addition of EGTA (2 mM), a calcium-chelator, in combination with adenosine deaminase, caused a large decline in basal and HA-responsive ^{3}H-cyclic AMP levels compared to incubation in their absence (Table 6). The decrease in basal activity was similar to that observed in the presence of adenosine deaminase alone (Table 6). In the presence of adenosine deaminase and EGTA, HA E_{max} values were reduced by 54.4% compared to incubation in the absence of these agents. However, since basal activity was reduced more than absolute HA-stimulation, fold HA stimulation was increased by a factor of 1.3. As with incubation with adenosine deaminase alone, ^{3}H-nucleotide levels were increased by about 10% in the presence of EGTA and adenosine deaminase relative to the absence of these agents and were uninfluenced by incubation with HA (not shown).

HA concentration-response curves appeared monophasic in both the absence and presence of combined adenosine deaminase plus EGTA. The slope indices of these curves were not significantly different from 1. EGTA in combination with adenosine deaminase, caused a shift in

the HA EC_{50} not significantly different to that seen with adenosine removal alone and both were significantly different from control incubations (Table 6; unblocked 1x ANOVA $p < 0.001$). Note however, that 2 experiments with adenosine deaminase plus EGTA, generating HA EC_{50} values of 55 and 110 uM, were excluded from this analysis.

2.3.2. Effect of Histamine-Receptor Antagonists on the Histamine Response.

In the presence of EGTA and adenosine deaminase, mepyramine (1 uM) had no effect on the HA response (Fig. 17). This observation is in contrast to the inhibition of the HA response by mepyramine in the absence of EGTA, when either adenosine deaminase was included (Fig. 14) or absent (Figs. 8 and 9).

In contrast to control incubations (Fig. 7), in the presence of EGTA and adenosine deaminase, cimetidine caused a concentration-dependent, parallel shift to the right in the response to HA ($F_{(3,17)} = 0.516$, $p > 0.05$, variance ratio test on a common slope fit to data shown in Fig. 18). Schild analysis of these data generated a slope not different from unity and a derived cimetidine pA_2 of 6.74 +/- 0.32. Thus, as with adenosine deaminase alone (Fig. 13), EGTA and adenosine deaminase removed the cimetidine-resistant component noted in the absence of these agents (Fig. 7). Furthermore, the cimetidine K_{bapp}

Fig. 17. Effect of EGTA and adenosine deaminase on mepyramine-inhibition of histamine-elicited accumulation of ^{3}H-cyclic AMP.

Vesicular preparations from 2 guinea pig cerebral cortices were prepared and incubated in 15 mM KRB, as described in Methods. Individual aliquots were incubated at 37 C in the absence (circle) or presence (triangle) of adenosine deaminase (2.5 U/ml). EGTA (2 mM) and mepyramine (1 uM) were added 20 min prior to incubation with HA, which was for 10 min. HA (●), HA + mepyramine (○), HA + EGTA (▲), HA + EGTA + mepyramine (△).

Each point represents the mean +/- SEM of 9 data points determined from 3 separate experiments, each in triplicate. Data are normalized (100%) to the control response obtained with 300 uM HA in each experiment. Curves shown represent logistic fits to the data points shown.

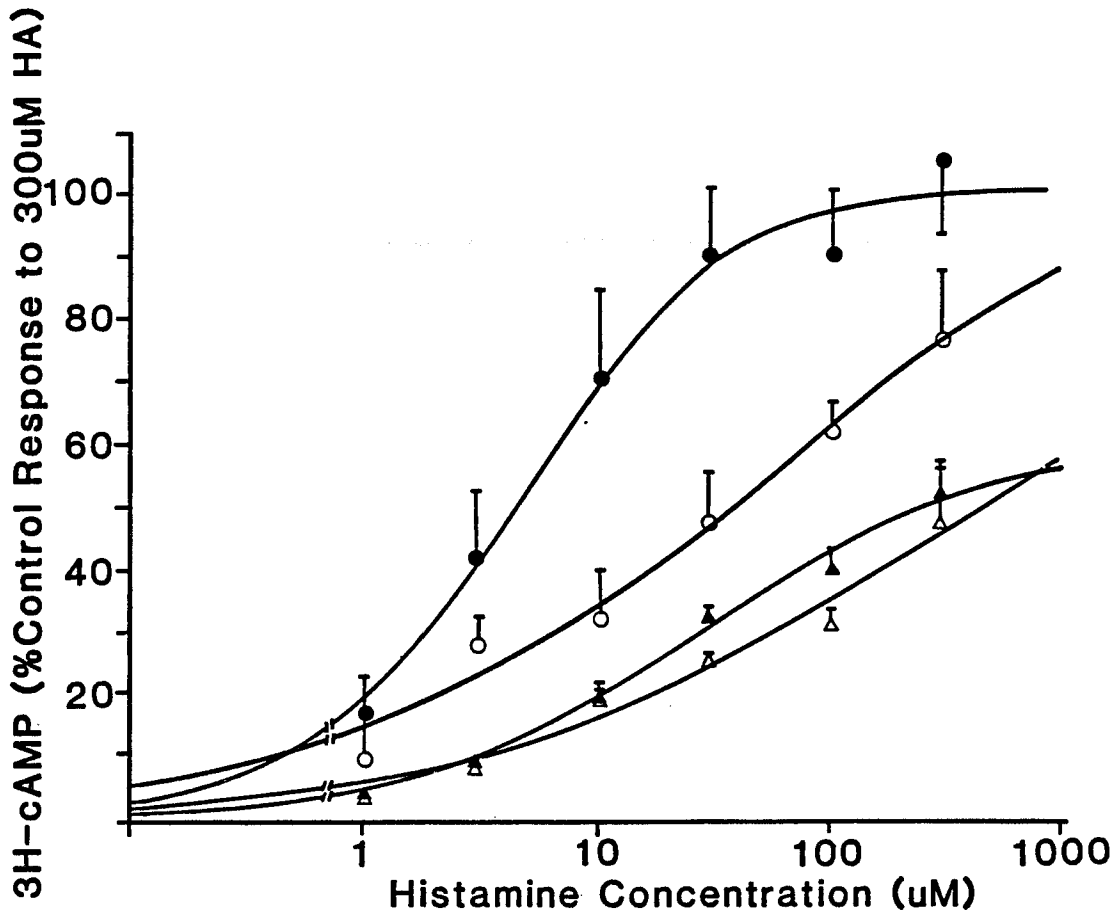

3H-cAMP (%Control Response to 300uM HA)
100
80
60
40
20
1
10
100
1000
Histamine Concentration (uM)

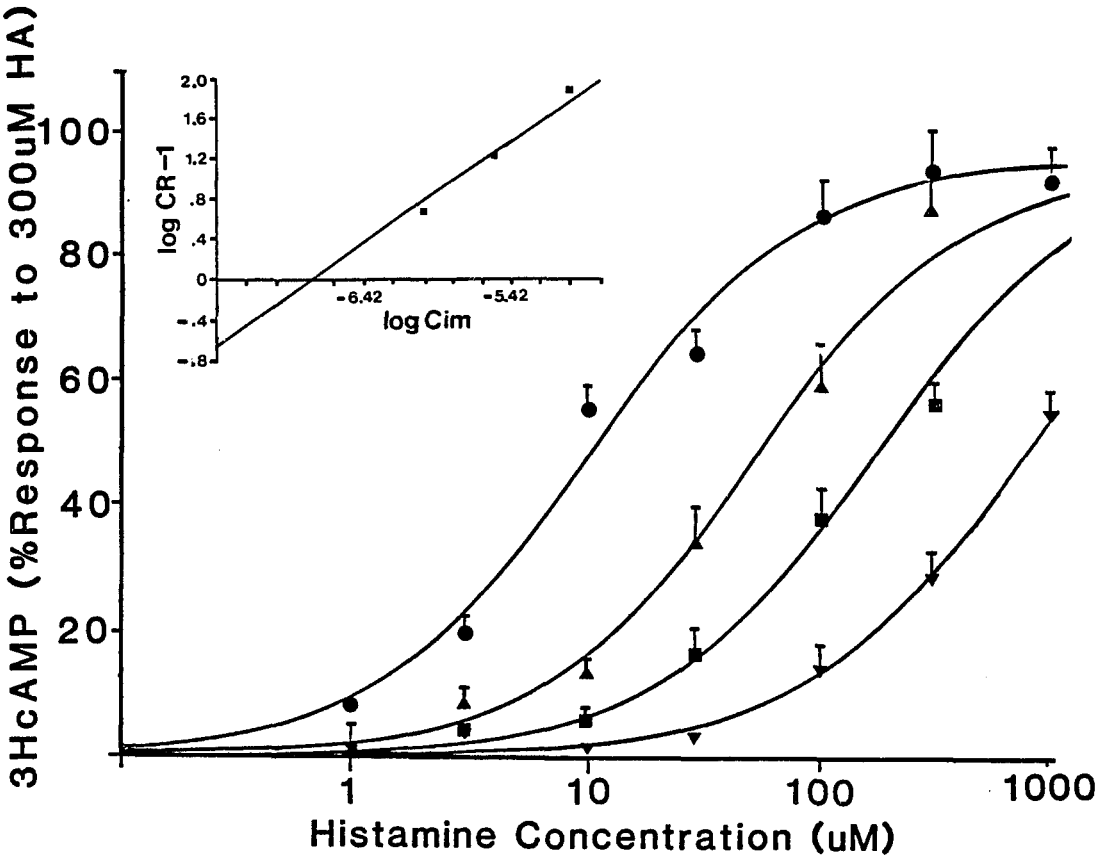
Fig. 18. Cimetidine antagonism of histamine-elicited accumulations of ^{3}H-cAMP in the presence of EGTA and adenosine deaminase.

Vesicular preparations were prepared from 2-3 guinea pig cortices in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the presence of adenosine deaminase (2.5 U/ml). Cimetidine [Ø (●), 1 (▲), 3 (■) and 1Ø (▼) uM] was added 2Ø min prior to incubation with HA, which was for 1Ø min.

Each antagonist curve represents the mean of 6 data points determined in 2 separate experiments, each in triplicate (control n=4). Data are normalized (1ØØ%) to the control 3ØØ uM HA response determined in each experiment. Curves shown represent common slope logistic fits to the data points shown.

Inset: Schild plot of data.

The regression of log [CR-1] vs log [cimetidine] results in a straight line with a slope 1.21 +/- Ø.2Ø, not significantly different from 1.Ø. The intercept of a line constrained to slope = 1.Ø (shown here) was used to determine the dissociation constant of cimetidine under these incubation conditions. The intercept (pA_2 = 6.74 +/- Ø.32) corresponds to a dissociation constant of Ø.18 uM.


3HcAMP (%Response to 300uM HA)
100
80
60
40
20
1
10
100
1000
Histamine Concentration (uM)
log CR-1
2.0
1.6
1.2
.8
.4
0
-.4
-.8
-6.42
-5.42
log Cim

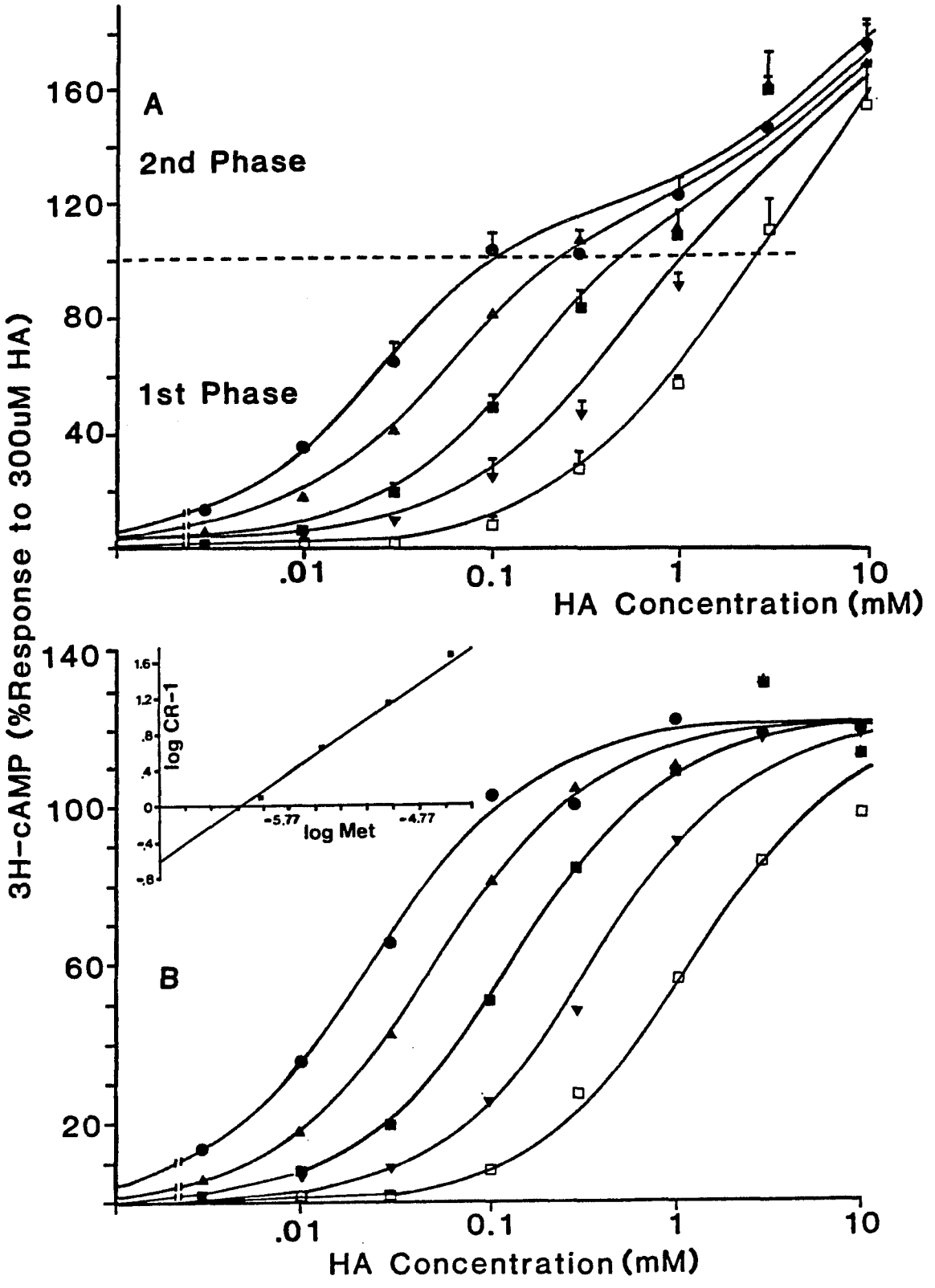
value is similar in both the absence or presence of adenosine deaminase plus EGTA (Ø.27 vs Ø.18 uM respectively) and strongly suggests that the H_2-receptor component of the HA response is similar under the 2 incubation conditions.

Experiments with another H_2-antagonist, metiamide, confirmed that adenosine deaminase and EGTA reduced the HA response to one mediated exclusively by H_2-receptor stimulation. This antagonist also caused concentration-dependent, surmountable changes in the HA concentration-response curve (Fig. 19). Increasing the concentration of HA above 1 mM caused an increase in ^{3}H-cyclic AMP levels above that associated with saturation of the HA concentration-response and did not appear to be influenced by metiamide (Fig. 19A). Subtracting this component of the HA response revealed that metiamide inhibition of the HA response was surmountable (Fig. 19B). No significant deviation from parallelism was found when HA concentration response curves were fitted to a common slope model ($F_{(4,28)} = 1.43$, $p > Ø.Ø5$, variance ratio test on a common slope fit to data shown in Fig. 19B), indicating that metiamide was acting as a competitive antagonist. Schild analysis of the data shown in Fig. 19B gave a slope not different from unity and a derived metiamide pA_2 of 6.15 +/- Ø.12 (Fig. 19 insert).

Fig. 19. Metiamide antagonism of the histamine response in the presence of EGTA and adenosine deaminase.

Vesicular preparations from 3 guinea pig cortices were prepared and incubated in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the presence of adenosine deaminase (2.5 U/ml). EGTA (2 mM) and metiamide [Ø (●), 1 (▲), 3 (■), 1Ø (▼) and 3Ø (□) uM] were added 2Ø min prior to incubation with HA, for 1Ø min. A. Each point represents the mean +/- SEM of 6 data points determined from 2 separate experiments, each in triplicate. Data are normalized (1ØØ%) to the stimulation obtained with 3ØØ uM HA in each experiment. HA concentration-response curves were divided into a saturable (1st phase) and a nonspecific (2nd phase) component by obtaining a logistic fit to control HA responses up to and including 1 mM. Curves shown were drawn by hand.

B. The observed increase in ^{3}H-cyclic AMP formation was corrected for a nonspecific component in the HA response (see A). Increased mean HA control values at 3 and 1Ø mM (25 and 55% respectively) were subtracted from all data points. Curves shown are common slope logistic fits to the resulting data points (see Methods for further details). Inset: Schild plot of data in B. The regression of log [CR-1] vs log [metiamide] results in a straight line with a slope (1.Ø9 +/- Ø.Ø3) not significantly different from 1. The pA_2 of metiamide (6.15 +/- Ø.12) was obtained from a line constrained to 1 (shown here).


A
2nd Phase
1st Phase
160
120
80
40
.01
0.1
1
10
HA Concentration (mM)
3H-cAMP (%Response to 300uM HA)
B
140
100
60
20
log CR-1
1.6
1.2
.8
.4
0
-.4
-.8
-5.77
-4.77
log Met
.01
0.1
1
10
HA Concentration (mM)

Tiotidine did not act as a competitive H_2-antagonist in this preparation (Fig. 20). In 3 separate experiments, 0.01 uM tiotidine caused a 50% reduction in the HA E_{max}, without causing significant effects on the HA EC_{50}. Increasing the concentration of tiotidine to 0.3, 1.0 or 3 uM produced further, but smaller, reductions in the HA E_{max}. At these concentrations tiotidine appeared to shift the HA EC_{50}. However, the remaining HA response was too small to allow accurate calculation of a pD_2 value.

2.3.3. Effect of Histamine-Receptor Agonists on ^{3}H-Cyclic AMP Accumulation.

The hypothesis that EGTA and adenosine deaminase eliminated an H_1-mediated component in the response to HA was supported by studies with additional HA-receptor agonists (Fig. 21). As noted above (Table 6), EGTA and adenosine deaminase caused a significant shift to the right in the HA concentration-response curve. In the presence of this combination, the concentration-response curve to the selective H_1-agonist TEA was also shifted to the right. The position of the dimaprit concentration-response curve was little influenced by this treatment. These observations suggest that an H_1-mediated process was abolished by adenosine deaminase and EGTA treatment and argue that this process contributes to the lower HA EC_{50} seen in control incubations.

Fig. 20. Tiotidine antagonism of the histamine response in the presence of EGTA and adenosine deaminase.

Vesicular preparations from 3 guinea pig cerebral cortices were prepared and incubated in 15 mM KRB, as described in Methods. Preparations were incubated at 37 C in the presence of adenosine deaminase (2.5 U/ml). EGTA (2 mM) and tiotidine [0 (●), 0.01 (■), 0.03 (○), 0.1 (▼) and 0.3 (△) uM] were added 20 min prior to incubation with HA, which was for 10 min.

Each point represents the mean +/- SEM of 9 data points determined from 3 separate experiments, each in triplicate. Data are normalized (100%) to the stimulation obtained with 300 uM HA in each experiment. Curves shown are logistic fits to the mean data points.

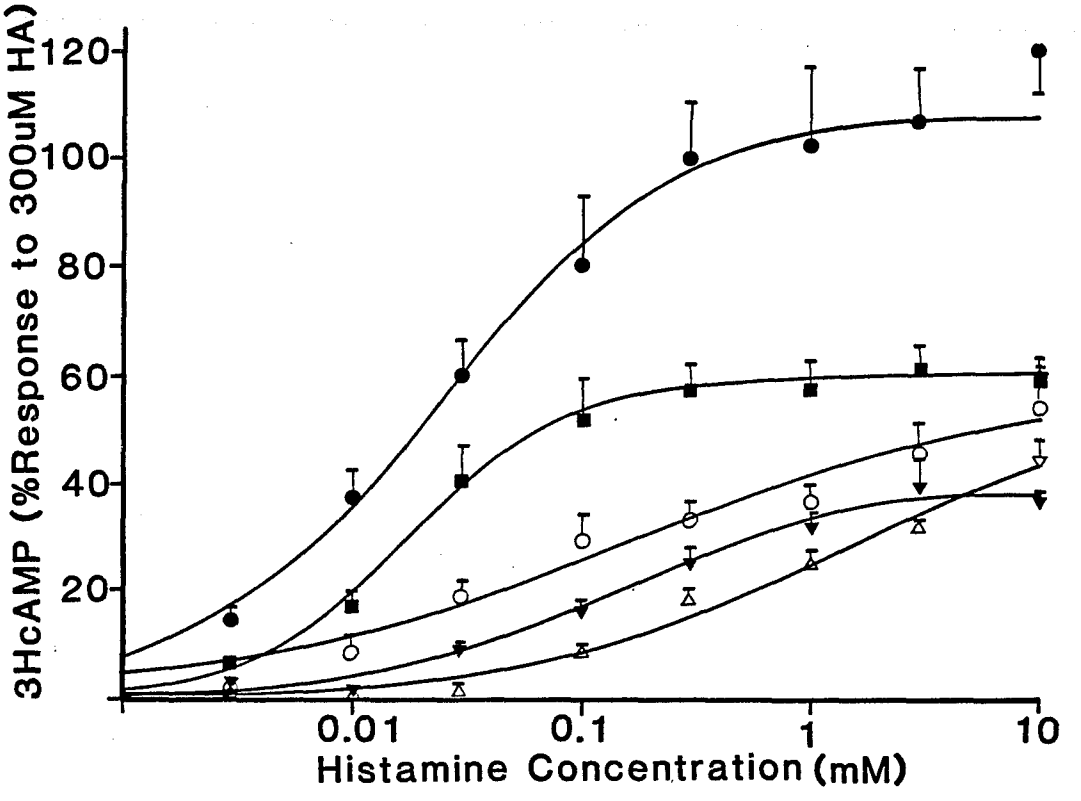
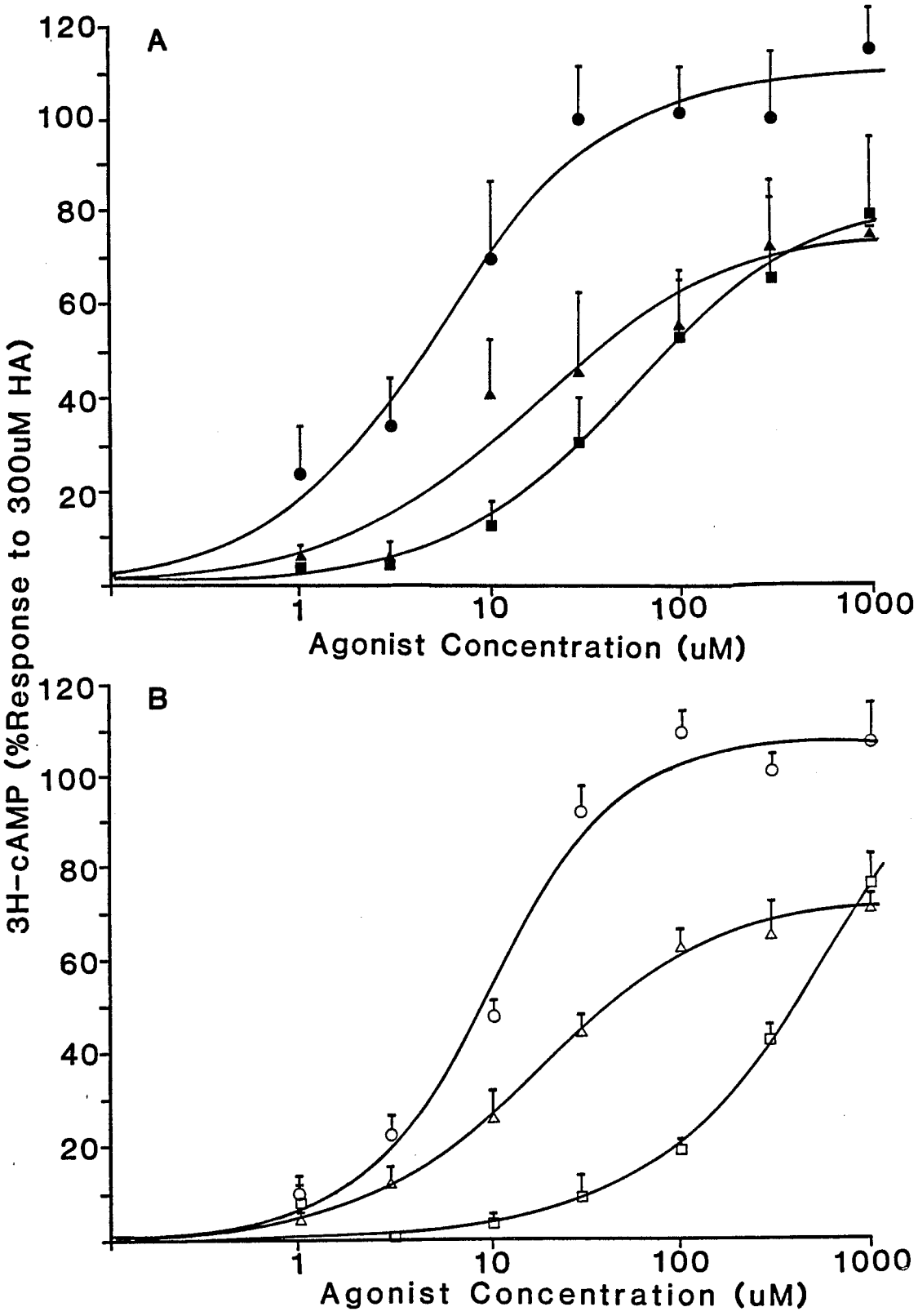

3HcAMP (%Response to 300uM HA)
120
100
80
60
40
20
0.01
0.1
1
10
Histamine Concentration (mM)

Fig. 21. Effect of EGTA and adenosine deaminase on the ^{3}H-cyclic AMP concentration-response curves to histamine-receptor agonists.

Vesicular preparations from 3 guinea pig cerebral cortices were prepared and incubated in 15 mM KRB, as described in Methods. Preparations were incubated at 37 C in the absence (A) or presence (B) of adenosine deaminase (2.5 U/ml). EGTA (B; 2 mM) or buffer (A) was added 20 min prior to incubation with HA (●,○), dimaprit (▲ , △) or TEA (■ , □), which was for 10 min.

Each point represents the mean +/- SEM of 6 data points determined from 2 separate experiments, each in triplicate. Both experiments gave similar results. Data are normalized (100%) to the response obtained with 300 uM HA under each incubation condition, determined in each experiment.

Curves are logistic fits to the data points shown.

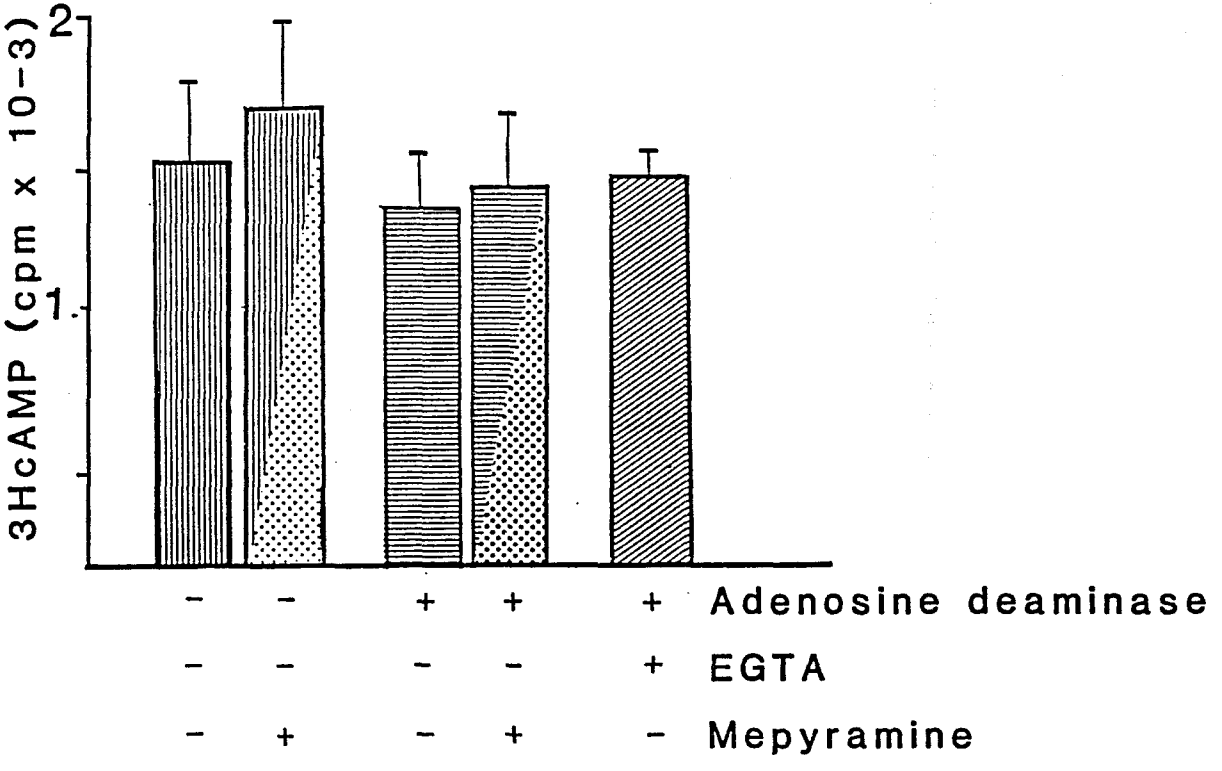

A
B
120
100
80
60
40
20
1
10
100
1000
3H-cAMP (%Response to 300uM HA)
Agonist Concentration (uM)
Agonist Concentration (uM)

Preliminary evidence suggests that the H_2-receptor contribution to HA-agonist mediated ^{3}H-cyclic AMP accumulation is not altered by adenosine deaminase with or without EGTA. In the 2 experiments summarized in Fig. 21 adenosine deaminase did not cause the activity of dimaprit to rise relative to HA, a predicted effect if H_1-mediated responses were abolished with concurrent maintenance of H_2-receptor mediated ^{3}H-cyclic AMP accumulation. However, when data from all experiments are combined (Fig. 22), it can be seen that the absolute response to 100 uM dimaprit does not change when adenosine deaminase, with or without EGTA, is included in the medium (Fig. 22).

Fig. 22. Comparison of the effect of adenosine deaminase, with or without EGTA, on dimaprit-stimulated ^{3}H-cyclic AMP levels.

Vesicular preparations from 2-3 guinea pig cerebral cortices were prepared in 15 mM KRB, as described in Methods. Incubation at 37 C was conducted in the absence or presence of adenosine deaminase (2.5 U/ml). EGTA (2 mM) or mepyramine (Ø.1 uM) was added 2Ø min prior to incubation with dimaprit (1ØØ uM), which was for 1Ø min.

Each bar represents the mean of 3 separate experiments, each determined in triplicate. Error bars represent the SEM of 3 x n data points. Data is taken from different experiments, only +/- mepyramine values are paired determinations.


3HcAMP (cpm x 10-3)
2
1
- - + + + Adenosine deaminase
- - - - + EGTA
- + - + - Mepyramine

2.4. Summary of Putative Histamine Receptors Mediating ^{3}H-Cyclic AMP Accumulation in the Vesicular Preparation.

A putative distribution of H_1- and H_2-receptors mediating ^{3}H-cyclic AMP accumulation in the vesicular preparation was constructed based on the maximum inhibition of the HA response by H_1- and H_2-receptor antagonists (Fig. 23). EC_{50} estimates for putative H_1- and H_2-receptor mediated components of the HA response were also established. In either the presence or absence of adenosine deaminase, based on the HA EC_{50} in the presence of 1 uM H_1-antagonists (see Figs. 8 and 14), the HA EC_{50} at H_2-receptors was estimated at 10 uM. In the absence of adenosine deaminase, based on the HA EC_{50} of the cimetidine-resistant component of the HA response (see Fig. 7), the HA EC_{50} at putative H_1-receptors was estimated at 3 uM. Similarly, in the presence of adenosine deaminase, based on the EC_{50} of HA-stimulated ^{3}H-cyclic AMP accumulation in the presence of dimaprit (Fig. 16), the HA EC_{50} at putative H_1-receptors was also estimated at 3 uM. A model encompassing these HA-mediated interactions and its relationship to the pharmacological results seen is developed in the following section.

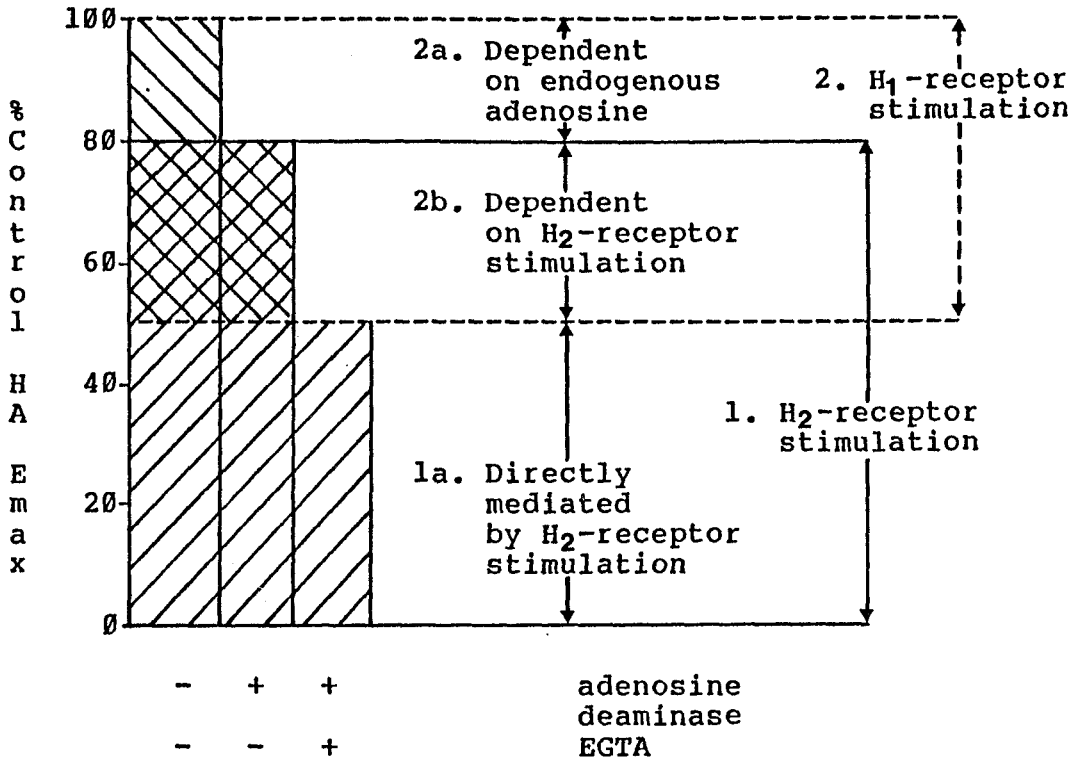


Fig. 23. Relative distribution of putative histamine receptors mediating histamine-stimulated ^{3}H-cyclic AMP accumulation in the vesicular preparation.

Schematic representation of the proportional distribution of putative HA receptors mediating the ^{3}H-cyclic AMP response to HA, in the absence or presence of adenosine deaminase with or without EGTA. The response to HA is normalized (100%) to the HA E_{max} (cpm) obtained in the absence of adenosine deaminase and EGTA (from Table 6). The maximum response to HA in the presence of adenosine deaminase with or without EGTA was also taken from Table 6. (continued on next page).

The dependency of the HA response on:

1. H_2-receptor stimulation was inferred from the maximum inhibition of this response by the H_2-antagonist cimetidine (Fig. 7, 13 and 18, in the absence or presence of adenosine deaminase without or with EGTA respectively).

1a. Direct H_2-receptor stimulation was attributed to that portion of the HA-response not inhibited by H_1-receptor antagonists (Fig. 9, 14C and 17 in the absence or presence of adenosine deaminase without or with EGTA respectively).

2. H_1-receptor stimulation was inferred from the maximum inhibition of this response by H_1-antagonists (Fig. 9, 14C and 17 in the absence or presence of adenosine deaminase without or with EGTA respectively).

2a. H_1-receptor stimulation dependent on endogenous adenosine for expression was inferred from the inability of cimetidine to abolish the HA-response in the absence of adenosine deaminase and EGTA (Fig. 7) and from the decrease in the sensitivity of the HA-response to inhibition by H_1-antagonists in the presence of adenosine deaminase compared to incubation in its absence (Fig. 14C vs 8 respectively).

2b. <u>H_1-receptor stimulation dependent on concommitent stimulation of H_2-receptors</u> was inferred from the observation that this fraction of the response is sensitive to inhibition by both H_1- and H_2-receptor antagonists in the absence (Fig. 7 and 8) or presence of adenosine deaminase (Fig. 13 and 17).

See Results 2.1. - 2.3. for further details.

3. Metactoid Sensitization as a Model for Histamine-Stimulated ^{3}H-Cyclic AMP Accumulation.

3.1. Description of Metactoid Sensitization.

The results presented suggest that both H_1- and H_2-receptors stimulate ^{3}H-cyclic AMP accumulation in the vesicular preparation. One potential model, predicting the agonist and antagonist results seen, is metactoid sensitization (Van Den Brink, 1977). According to this model (shown schematically in Fig. 24) H_2-receptor activation (R_a) will directly stimulate adenylate cyclase and hence cyclic AMP synthesis (E_a). H_1-receptor stimulation (R_b) does not find expression in a directly measurable effect (i.e. cyclic AMP) but acts indirectly (E_b) to potentiate the action of H_2-receptor stimulation. This potentiation is directly proportional to H_1-receptor stimulation. Under this model, H_1-receptor activation would be classed as acting through metactoid sensitization (Van Den Brink, 1977). This model is analogous to a pure V_{max} allosteric model in enzymology (Van Den Brink, 1977). The original definition of metactoid interactions (Van Den Brink, 1977) stipulates that the metactoid interaction occurs at a step distal to the receptors under study. In this study, the term metactoid sensitization is used to imply any indirect stimulus that would be encompassed by, but not restricted to, a system exhibiting metactoid sensitization as currently defined.

Fig. 24 Schematic representation of a model exhibiting metactoid sensitization.

Shown is a schematic representation of extracellular receptors (R_a, R_b and R_c) coupled to increased intracellular cyclic AMP levels. D acts as an agonist at both R_a and R_b, but not R_c. C acts as an agonist only at R_c. In this example, occupancy of R_a or R_c directly increases cyclic AMP levels through E_a or E_c respectively. Occupancy of R_b by agonist D does not directly increase cyclic AMP but acts, through an unknown effector mechanism (E_b) to potentiate the cyclic AMP response to directly acting stimuli (i.e. R_a and R_c). Conceptually, this potentiation might occur through stimulation of cyclic AMP synthesis (------).

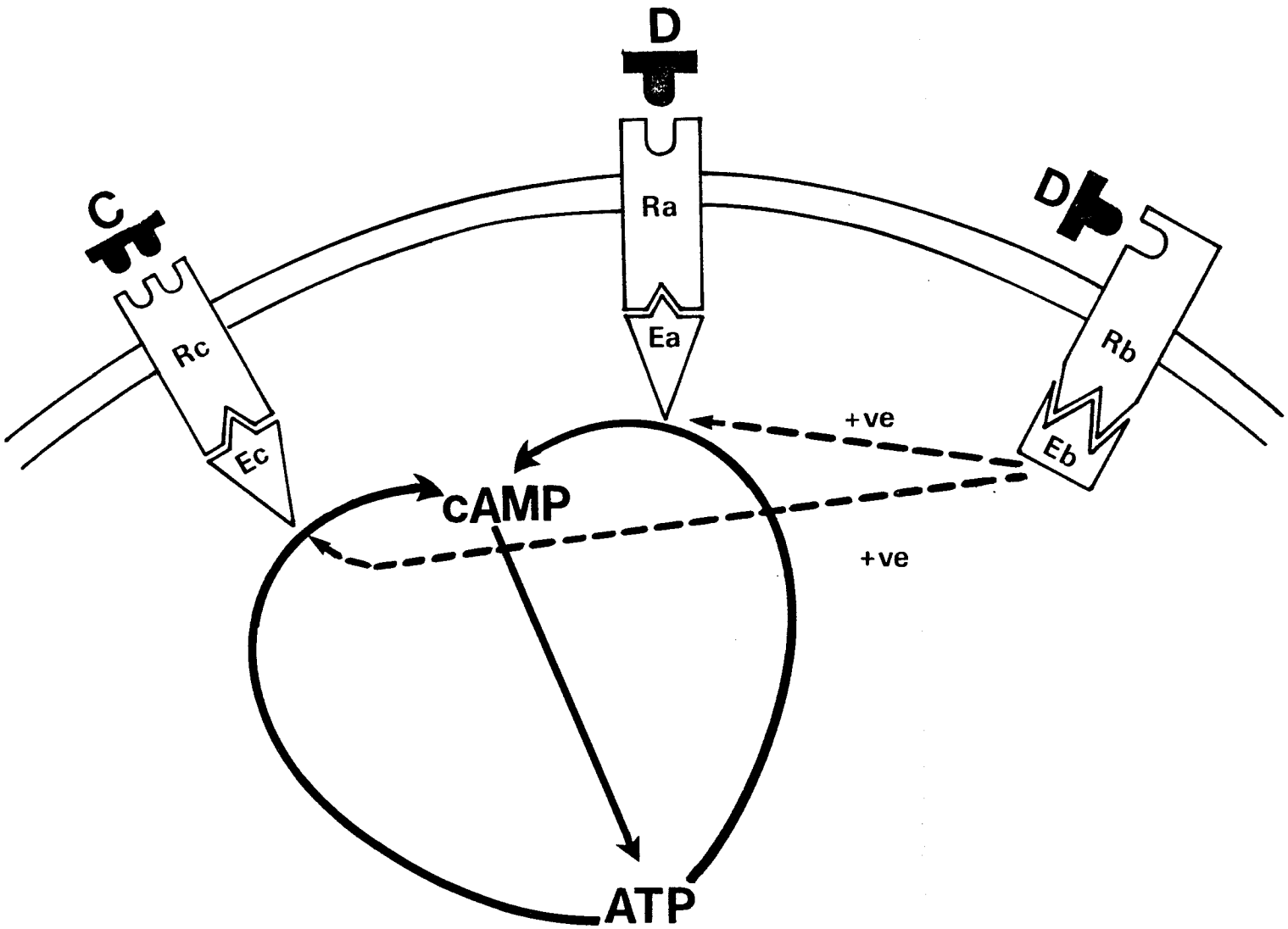
For the present results:

D = HA C = adenosine

R_a = H_2-receptors R_c = adenosine-receptors

R_b = H_1-receptors

See text for further details.


D
C
Ra
Rc
Ea
Ec
Rb
Eb
+ve
cAMP
+ve
ATP

3.2. Development and Simulations of the Metactoid Sensitization Model.

3.2.1. Potentiation of the Response to R_a by R_b:

The equation describing the interaction of a metactoid sensitizer (D) potentiating the response to a single directly acting agonist (in this instance, also D) is (after Van Den Brink, 1977):

$$\text{Response to D} = \frac{E_{ab}}{E_{abmax}} = \frac{E_a(1 + (p-1)E_b)}{E_{abmax}} \qquad (1)$$

where:

E_a = response to D at R_a

E_b = response to D at R_b

E_{ab} = response to any combination of E_a and E_b

E_{abmax} = maximum response of E_a (E_{amax}) in the presence of the maximum response to E_b (E_{bmax}).

$= E_{amax}(1 + (p-1)E_{bmax})$

p = system constant to describe the nature of interaction of E_a and E_b, e.g.:

$p = 1$ response is due to E_a alone

$p > 1$ E_b potentiates the response to E_a (E_b = metactoid sensitizer)

$p < 1$ E_b inhibits the response to E_a (E_b = metactoid inhibitor)

(Note that the equation describing a metactoid sensitizer or inhibitor is the same and only the value of p determines the effect of the metactoid agent. In the model formulated below, only metactoid sensitization is considered, i.e. $p > 1$).

Assuming:

1. The responses to E_a and E_b are directly proportional to occupancy of R_a and R_b respectively, i.e. no spare receptors.
2. The responses to E_a and E_b occur as separate events ie. the response to E_a alone is not influenced by E_b and vice versa.
3. D acts as a full agonist at both E_a and E_b.
4. All reacting species are at equilibrium.

Equation 1 becomes:

$$\text{Response to D} = \left[\frac{[D]}{[D] + K_a}\right]\left[1 + \frac{(p-1)\,[D]}{[D] + K_b}\right] \quad (2)$$

where:

[D] = agonist concentration

K_a = dissociation constant of D at R_a

K_b = dissociation constant of D at R_b

The shape of the resulting curve can be seen (from Equation 2) to be a function of variations in p, the concentration range over which D is studied and the

relationship between the dissociation constants of D at R_a (K_a) and R_b (K_b).

It can be seen from equation 2 that when R_b is unoccupied by D ($K_b >> K_a$), only the response to E_a, will be seen (see Fig. 25 $K_b = \infty$) i.e. there is no metactoid interaction. When R_b is fully occupied prior to R_a ($K_b << K_a$) the response to E_b is expressed only as an increase in the maximum effect of D, determined by the value of p (see Fig. 25 $K_b = \emptyset.\emptyset 1 K_a$). Thus at either of these two extremes, 50% of the maximum effect occurs at K_a.

Introducing competitive antagonists A and B acting exclusively at R_a and R_b respectively, Equation 2 becomes:

$$\text{Response to D} = \left[\frac{[D]}{[D] + K_a(1 + [A]/K_i)}\right]\left[1 + \frac{(p-1)\ [D]}{[D] + K_b(1 + [B]/K_{i'})}\right] \quad (3)$$

where:

[A] = antagonist concentration, acting at R_a.

K_i = dissociation constant of A at R_a

[B] = antagonist concentration, acting at R_b

$K_{i'}$ = dissociation constant of B at R_b

The effect of the initial values of K_b and K_a on the pattern of inhibition of a competitive antagonist (A) acting at R_a is shown in Fig. 26. When $K_b << K_a$ (D will fully occupy R_b prior to R_a) increasing concentrations of A will shift the response to D to the right in a fashion

Fig. 25. Simulation of metactoid sensitization - Effect of varying K_b/K_a on the response to D when only R_a can serve as the direct stimulus.

Theoretical concentration-response curves to an agonist D based on equation 2 of the metactoid model. In this model, D initiates the response through activation of 2 receptors (R_a and R_b). Occupancy of R_a by D directly initiates a response (E_a), while occupancy of R_b potentiates this response, by the amount $E_a \times E_b$, but cannot itself directly elicit this response.

Shown is the effect of varying K_a/K_b on the response to D (which is expressed in units of K_a on the x-axis and normalized (100%) to the maximum response to D). Each curve is labeled with the value of K_b/K_a for that curve in A. The curves in B have the same K_b/K_a values as those labeled in A.

A. p = 3.333

(This p value is equivalent to 30% of the response to D being mediated through Epfigleg

a and 70% through the

interaction of E_b on E_a).

B. p = 1.43

(This p value is equivalent to 70% of the response to D being mediated through E_a and 30% through the interaction of E_b on E_a).

See text for further details.

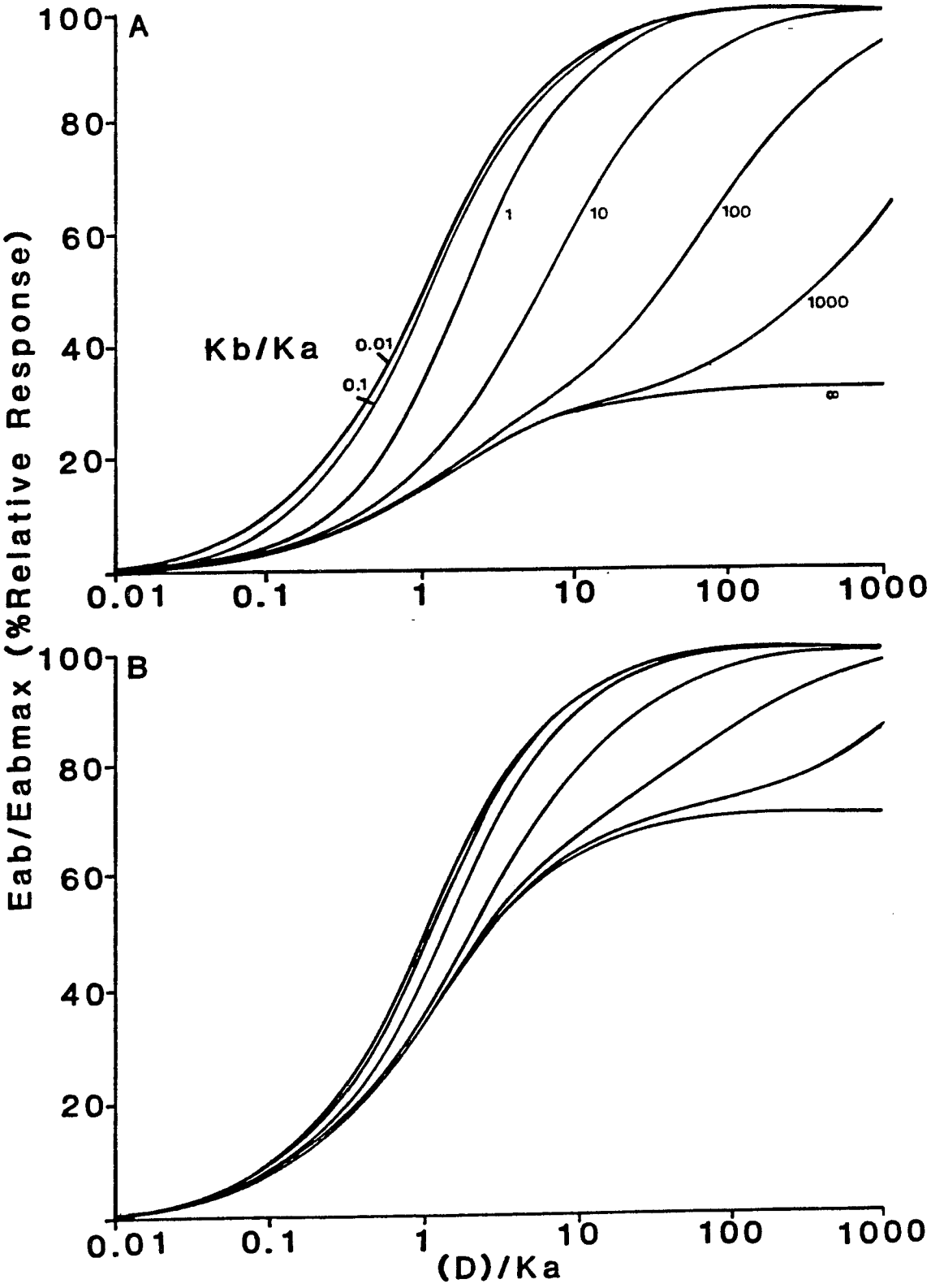
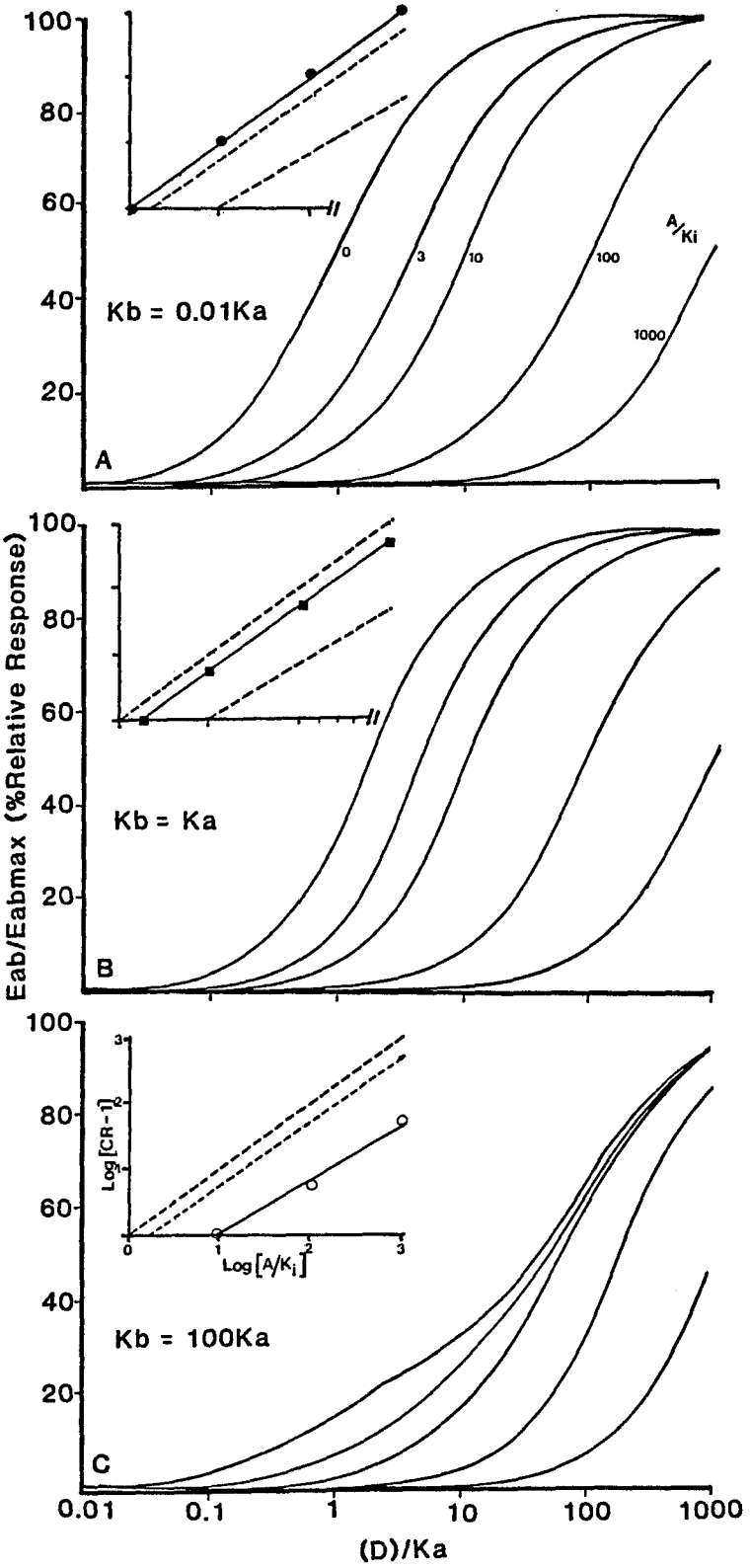

A
B
100
80
60
40
20
Eab/Eabmax (%Relative Response)
Kb/Ka
0.01
0.1
1
10
100
1000
∞
0.01
0.1
1
10
100
1000
(D)/Ka

Fig. 26. Simulation of metactoid sensitization - Effect of varying K_b/K_a on the pattern of inhibition seen with a competitive antagonist A, acting at R_a when only R_a can serve as the direct stimulus.

Theoretical concentration-response curves to an agonist D, based on equation 3 of the metactoid model. D initiates the response through activation of 2 receptors (R_a and R_b). Occupancy of R_a by D initiates a response (E_a). Occupancy of R_b potentiates this response by the amount $E_a \times E_b$, but R_b cannot directly elicit this response. An antagonist A (with dissociation constant K_i) can act to inhibit the response to D at R_a, but not R_b.

Shown is the effect of increasing concentrations of A (in units of A/K_i, as given in A), for selected K_a/K_b ratios (A-C, A/Ki is the same for all panels), on the response to D (expressed in units of K_a and normalized (100%) to the maximum response to D). In this example, p = 1.43 (see legend to Fig. 25).

Inset: Schild plots obtained by fitting simulated data points (27 points for each curve, evenly distributed across the concentration-range shown) to a common slope model (see Methods). Solid lines show the Schild plots obtained for the K_b/K_a ratio shown in the same panel. Dotted lines represent Schild plots obtained at other K_b/K_a ratios, and are included for comparison.
See text for further details.


Eab/Eabmax (%Relative Response)
100
80
60
40
20
A
Kb = 0.01Ka
0
3
10
100
1000
A/Ki
B
Kb = Ka
C
Kb = 100Ka
Log [CR - 1]
Log [A/Ki]
0.01
0.1
1
10
100
1000
(D)/Ka

indistinguishable from simple competitive antagonism, i.e. neither the response to E_a alone or the sensitization of E_a by E_b will be revealed (see Fig. 26A; K_b = Ø.Ø1 K_a). Actually, when K_b = Ø.Ø1 K_a the theoretical curves do significantly deviate from parallelism, (since D will occupy R_b prior to full occupancy of R_a), but it is unlikely that these deviations from simple competitive antagonism could be practically determined. Indeed, only when K_b is much larger that K_a (D will occupy R_a prior to R_b) will the characteristics of this model be revealed by increasing concentrations of A and/or the biphasic shape of the curve in response to D (see Fig. 26; A/K_i = Ø; A-D). This can be clearly seen in Fig. 26C (K_b = 1ØØ K_a). In this example, low concentrations of A will shift the response to E_a alone to the right. With increasing concentrations of D, the response attributable to the interaction of E_b on E_a will also be expressed. At this point, the effect of A at R_a is already surmounted, such that this response is not influenced by A. With high concentrations of A, both the response to D at E_a and the response to the interaction of E_b on E_a are inhibited (see Fig. 26C). This is seen as a simultaneous shift to the right of the response to E_a and (E_a) x (E_b). Only when R_b is either fully occupied or unoccupied will the pA_2 of A be accurately assessed (see Fig. 26 inset). Between these extremes, the pA_2 of A will be underestimated.

The effect of varying the ratio of K_b/K_a on the response to D is also shown in Fig. 25. Note that changes in this ratio alter the pattern of inhibition of a competitive antagonist B acting at R_b. As noted above, when D has very low affinity at R_b relative to R_a, only the response to E_a is seen; when D has very high affinity at R_b relative to R_a the effect of E_b on E_a is maximal (see Fig. 25A, $K_b = 0.01\ K_a$ vs $K_b = \infty\ K_a$). Because the response to D is obtained from occupancy of R_a, occupancy of R_b prior to R_a does not shift the concentration-response curve to D to the left (see Fig. 25A, $K_b = 0.01\ K_a$ vs $K_b = 0.1\ K_a$). The difference between K_b and K_a thus determines the effect of a competitive antagonist B acting at R_b on the response to D (see Fig. 25A as K_b goes from 0.01 to 0.1 K_a vs from 1 to 10 K_a).

When the proportion of direct to indirect components of the response to D is increased (ie. the value of p is reduced), the effect of E_b on the overall response is decreased (Fig. 25A vs B). Note that in either case when R_b is unoccupied or fully occupied (relative to R_a), the half maximal effect of D occurs at K_a.

3.2.2. Potentiation of the Response to R_a and R_c by R_b.

The simulations described above have similarities with the ^{3}H-cyclic AMP response to HA in the presence of adenosine deaminase (see Results 3.2.). However, in

the absence of adenosine deaminase, the present findings cannot be accounted for by this model (see Discussion 2.). Therefore another receptor (R_c) directly coupled to cyclic AMP accumulation (E_c), through which stimulation of R_b can also act as a metactoid sensitizer, must also be introduced (see Fig. 24).

Incorporating the assumptions that:

1. Occupancy of another independent receptor R_c by agonist C can also directly stimulate cyclic AMP accumulation.
2. D has no affinity for R_c and C has no affinity for R_a or R_b.
3. Stimulation of R_b can act as a metactoid sensitizer by potentiating the response to both E_a and E_c.
4. The response to E_a and E_c are additive.
5. The response to the interaction of E_b on E_a is not altered by the interaction of E_b on E_c and vice versa.

The overall response (from equation 1) becomes:

$$\text{Overall response} = \frac{E_{abc}}{E_{abcmax}} = \frac{E_{ab} + E_{bc}}{E_{abcmax}} \qquad (4)$$

where:

E_{bc} = response to any combination of E_c and E_b.

E_{abc} = response to any combination of E_a, E_b and E_c.

E_{abcmax} = maximum response to E_a and E_c in the presence of the maximum response to E_b.

The equation describing the metactoid interaction of E_b on E_c (analagous to equation 1) is:

$$\frac{E_{bc}}{E_{abcmax}} = \frac{E_c\ (1 + (q-1)E_b)}{E_{abcmax}} \qquad (5)$$

where:

E_c = response to C at R_c.

q = system constant to describe the nature of the interaction of E_c and E_b. (Analagous to p in equation 1).

Combining equations 1 and 5:

$$\text{Overall response} = \frac{E_a(1 + (p-1)E_b) + E_c(1 + (q-1)E_b)}{E_{abcmax}} \qquad (6)$$

To develop the relationship of agonist D in this model, I considered the response to D in the presence of a fixed concentration of C. From equation 6:

$$\text{Response to D} = \frac{E_a(1 + (p-1)E_b) + E_c(q-1)E_b}{E_{abcmax}} \qquad (7)$$

introducing r as:

$$r = E_c(q-1) \qquad (7a)$$

and substituting into equation 7 gives:

$$\text{Response to D} = \frac{E_a(1 + (p-1)E_b) + rE_b}{E_{abcmax}} \qquad (8)$$

Incorporating previously defined assumptions, we obtain:

$$\text{Response to D} = \left[\frac{[D]}{[D] + K_a(1 + [A]/K_i)}\right]\left[1 + \frac{(p-1)[D]}{[D] + K_b(1 + [B]/K_{i'})}\right] + \left[\frac{r[D]}{[D] + K_b(1 + [B]/K_{i'})}\right] \quad (9)$$

Under these conditions, the response to E_b is no longer dependent on E_a for expression. In the presence of a fixed concentration of C, the overall response to D will depend on the ratios p/r and K_a/K_b.

In contrast to incubation with D alone, in the presence of a fixed concentration of C, increasing concentrations of A will reveal the extent of the interaction of E_b on E_c (Fig. 27A). This is produced by the simultaneous shift to the right of the response to E_a and the response to the interaction of E_b on E_a (see Results 3.1.1.). The concentration of D at which the interaction of E_b on E_c reaches half its maximum should equal K_b. It is apparent from Equation 9 that, under the assumptions given, the dependency of the curve on K_b/K_a (as discussed above) does not affect the position of that component of the curve showing the interaction of E_b on E_c.

In the presence of a fixed concentration of C, the effect of E_b on E_c is also revealed (Fig. 27B). When K_b/K_a is small (occupancy of R_b by D is greater than the occupancy of R_a) the effect of E_b on E_c can be seen as a response obtained at concentrations of D not active in the absence of E_c. This is because in the absence of E_c the response to E_b is entirely dependent on E_a, and hence K_a; while in the presence of E_c, the response to the interaction of E_b on E_c, which is dependent on r and K_b, is also observed. Within the model of an E_a response independent of E_c, this difference is apparent from a comparison of Fig. 25B and 27B.

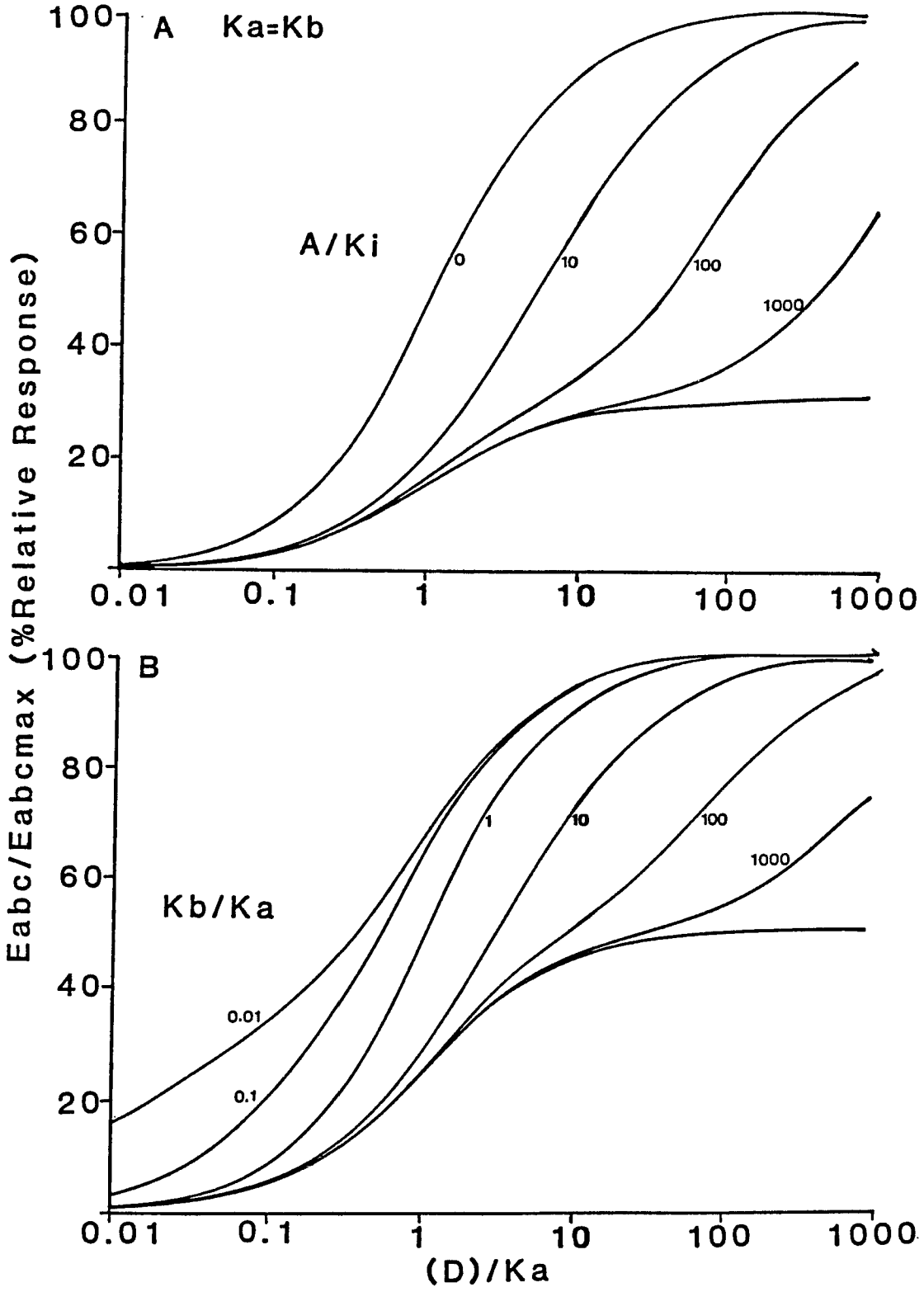
Fig. 27. Simulation of metactoid sensitization - Effect of varying K_a/K_b on the response to D when both R_a and R_c can serve as the direct stimulus.

Theoretical concentration-response curves to agonist D, based on equation 9 of the metactoid model. D initiates the response through activation of 2 receptors (R_a and R_b). Occupancy of R_a by D initiates a response (E_a). Occupancy of R_b cannot itself directly elicit this response but potentiates the response to E_a, by the amount $E_a \times E_b$, and potentiates the response to another directly acting agonist C (present at a fixed concentration) by the amount $E_b \times E_c$. In this example, p = 1.40 and r = 0.6 (see equation 9); these constants are equivalent to 50% of the maximum response to D being mediated through E_a, 20% through the interaction of E_b on E_a and 30% through the interaction of E_b on E_c.

Shown is the effect of varying K_b/K_a on the response to D (expressed in units of K_a on x-axis and normalized (100%) to the maximum response to D) for:

A. Decreasing the apparent K_b/K_a ratio by addition of a competitive antagonist A (in units of A/K_i as labeled in A), acting exclusively at R_a, when $K_b/K_a = 1$.

B. Varying K_b/K_a (in units of K_b/K_a as labeled in B). Note that increasing K_b/K_a predicts the pattern of inhibition obtained by addition of antagonist B, acting exclusively at R_b. See text for further details.


A Ka=Kb
A/Ki
0
10
100
1000
B
Kb/Ka
0.01
0.1
1
10
100
1000
Eabc/Eabcmax (%Relative Response)
(D)/Ka
100
80
60
40
20
0.01
0.1
1
10
100
1000

3.3. Fit of Histamine Concentration-Response Curves to the Metactoid Sensitization Model.

Curve fitting of the data to the metactoid model was conducted by use of estimates of p and r determined from the cimetidine and H_1-antagonist insensitive proportions of the HA response (Fig. 23). Thus, 55% of the control HA response (Ea) was attributed to direct stimulation of H_2-receptors (R_a); 25% of the control HA response (E_{ab}) was attributed to putative H_1-receptor stimulation (R_b) which was dependent on H_2-receptor stimulation; 20% of the control HA response ((E_{bc}) was attributed to putative H_1-receptor stimulation which was dependent on endogenous adenosine. In the presence of adenosine deaminase, 69% of the HA response was attributed to direct stimulation of H_2-receptors and 31% to putative H_1-receptor stimulation dependent on H_2-receptor stimulation. Thus, from equation 9, p = 1.45 in either the presence or absence of adenosine deaminase, and r = Ø or Ø.36 in the presence or absence of adenosine deaminase, respectively (see legend to Table 7).

In the presence of adenosine deaminase and EGTA, since no H_1-antagonist-sensitive component was apparent, p = 1 and r = Ø, i.e. the response is mediated only through direct H_2-receptor activation. This latter condition was therefore not included in the curve fitting procedures. Note that within the simple occupancy assumptions of the

metactoid model presented, K_a (the dissociation constant for HA at H_2-receptors) should equal the EC_{50} of this response which is 18 uM (Table 6).

Initial estimates of the HA dissociation constants at H_2- (K_a) and H_1-receptors (K_b) were obtained from fitting the mean HA concentration-response curves (Table 6), in the presence and absence of adenosine deaminase, to a modified version of Equation 9 of the metactoid sensitization model (see legend to Table 7). With p and r fixed, a series of fits to K_b were performed by varying initial estimates of K_a (Table 7). In both the absence and presence of adenosine deaminase, increasing K_a leads to a decrease in fitted K_b values while decreasing K_a leads to an increase in fitted K_b values (Table 7). Relative to incubation in the absence of adenosine deaminase, the larger change in fitted K_b values with varying K_a in the presence of adenosine deaminase is presumably attributable to the additional constraint imposed on the fitting procedure due to removal of the putative H_1-component independent of H_2-receptor stimulation (E_{bc}). In the presence of adenosine deaminase, the negative fitted values of K_b when K_a was increased above 10 uM (Table 7) are also a result of removal of E_{bc} from the overall response to HA. In contrast to control studies where the best fit was obtained when K_a was set to 6 uM with a fitted K_b of

Table 7. Stepwise fitting of histamine concentration-response curves to the Metactoid Sensitization Model.

A. Incubations in the absence of adenosine deaminase and EGTA.

Fitted data from	Antagonist	Parameters fit to the Metactoid Sensitization Model. p	r	Ka (uM)	Kb (uM)	K*** (nM)	SS	Comments
Table 6 (c)	none	1.45 (a)	0.36 (a)	1.00 (a)	20.10 +/- 7.01 (b)	-	1050	
		"	"	3.00 (a)	8.68 +/- 0.98 (b)	-	85	
		"	"	6.00 (a)	*2.62 +/- 0.26 (b)	-	35	Best combination fit at Ka = 6 uM (lowest SS).
		"	"	10.00 (a)	0.89 +/- 0.33 (b)	-	264	
		"	"	30.00 (a)	0.35 +/- 0.60 (b)	-	3313	
		"	"	4.88 +/- 23.3 (b)	4.01 +/- 41.00 (b)	-	25	Confirms best estimate of Ka and Kb in uM range but S.D. large.
Fig. 7A	cimetidine	"	"	10.00 (a)	1.21 +/- 0.34 (b)	478 +/- 56 (b)	794	
		"	"	6.00 (a)	*2.05 +/- 0.43 (b)	280 +/- 27 (b)	576	Confirms best combination fit at Ka = 6 uM (p<0.05, SS at Ka = 10 uM t-test n = 4).
Fig. 8A	mepyramine	"	"	10.00 (a)	1.18 +/- 0.08 (b)	0.53 +/- 0.41 (b)	1705	
		"	"	6.00 (a)	*3.47 +/- 0.15 (b)	1.02 +/- 0.52 (b)	1203	
Fig. 8B	d-chlorphen -iramine	"	"	10.00 (a)	0.73 +/- 0.77 (b)	0.10 +/- 0.11 (b)	1266	
		"	"	6.00 (a)	*2.51 +/- 0.14 (b)	0.22 +/- 0.15 (b)	892	
					2.67 +/- 0.30			Best fitted estimate of Kb at Ka = 6 uM (mean +/- SEM n = 4 (*)).
Table 6	-	"	"	**5.77 +/- 0.21 (b)	2.67 (a)	-	33	
Fig. 7A	cimetidine	"	"	**5.37 +/- 0.68 (b)	"	261 +/- 43 (b)	592	Confirms initial best estimate of Ka
Fig. 8A	mepyramine	"	"	**6.24 +/- 0.73 (b)	"	0.79 +/- 0.22 (b)	1014	
Fig. 8B	d-chlorphen -iramine	"	"	**5.01 +/- 0.73 (b)	"	0.38 +/- 0.13 (b)	886	
				5.60 +/- 0.26				Best fitted estimate of Ka at Kb = 2.67 uM (mean +/- SEM n = 4 (**)).
Fig. 7A	cimetidine	"	"	5.60 (a)	"	271 +/- 25	593	
Fig. 8A 9	mepyramine	"	"	"	"	0.74 +/- 0.20 (b)	1248	Best fitted estimate of antagonist dissociation constants.
		"	"	"	"	0.84 +/- 0.20 (b)	48	
Fig. 8B 9	d-chlorphen -iramine	"	"	"	"	0.40 +/- 0.14 (b)	910	
		"	"	"		0.30 +/- 0.09 (b)	114	
Fig. 9	l-chlorphen -iramine	"	"	"	"	18.06 +/- 3.74 (b)	77	

		p	r	Ka	Kb	K***	SS	
Fig. 7A	cimetidine	1.44 +/- 0.05 (b)	0.38 +/- 0.03 (b)	5.60 (a)	2.67 (a)	271 (a)	0.19	
Fig. 8A	mepyramine	1.56 +/- 0.20 (b)	0.24 +/- 0.17 (b)	"	"	0.74 (a)	0.40	
Fig. 9	d-chlorphen -iramine	1.30 +/- 0.28 (b)	0.42 +/- 0.25 (b)	"	"	0.40 (a)	0.31	Confirms initial best estimates of p and r. (mean +/- SEM n = 3)
		1.43 +/- 0.08	0.35 +/- 0.05					

(Table 7. continued)

B. Incubations in the presence of adenosine deaminase.

Fitted data from	Antagonist	Parameters fit to the Metactoid Sensitization Model					
		p	Ka (uM)	Kb (uM)	K*** (nM)	SS	Comments
Table 6 (c)		1.45 (a)	1.00 (a)	88.50 +/- 89.60 (b)	-	4346	
		"	3.00 (a)	51.95 +/- 25.80 (b)	-	924	
		"	6.00 (a)	21.60 +/- 3.72 (b)	-	76	
		"	10.00 (a)	2.43 +/- 0.13 (b)	-	1	Best combination fit at
		"	11.75 (a)	- 0.43 +/- 0.05 (b)	-	13	Ka = 10 uM (lowest SS).
		"	30.00 (a)	- 4.87 +/- 0.46 (b)	-	2045	
Fig. 29B	mepyramine	"	10.00 (a)	2.31 +/- 2.96 (b)	0.43 +/- 0.57 (a)	487	In all cases the large error
		"	6.00 (a)	25.49 +/- 14.00 (b)	3.28 +/- 2.43 (a)	671	in Kb is due to experimental variation in the H1-antagonist
Fig. 14	d-chlorphen -iramine	"	10.00 (a)	0.95 +/- 3.15 (b)	0.25 +/- 0.84 (a)	899	sensitive component (since
		"	6.00 (a)	16.33 +/- 11.57 (b)	3.61 +/- 3.16 (a)	657	this is very small under these incubation conditions)
Fig. 14	promethazine	"	10.00 (a)	0.92 +/- 2.42 (b)	0.35 +/- 0.94 (a)	536	All curves in the absence of
			6.00 (a)	16.15 +/- 11.57 (b)	4.97 +/- 4.30 (a)	660	antagonist were better fit at Ka = 10 uM vs 6 uM.
Fig. 15	doxepin	"	10.00 (a)	1.14 +/- 1.86 (b)	0.13 +/- 0.22 (a)	223	
			6.00 (a)	15.67 +/- 8.10 (b)	1.04 +/- 0.82 (a)	368	
Fig. 29B	mepyramine	"	10.00 (a)	2.67 (a)	0.51 +/- 0.15 (a)	489	Best fitted estimates of antagonist dissociation
Fig. 14	d-chlorphen	"	"	" (a)	0.79 +/- 0.43 (a)	905	constants, in the presence of adenosine deaminase.
Fig. 14	promethazine	"	"	" (a)	1.05 +/- 0.43 (a)	538	Similar to values obtained in the absence of adenosine
Fig. 15	doxepin	"	"	" (a)	0.31 +/- 0.10 (a)	475	deaminase.
Fig. 29B	mepyramine	1.42 +/- 0.03 (b)	10.00 (a)	2.67 (a)	0.51 (a)	0.10	Confirms initial estimate of
Fig. 14	d-chlorphen	1.46 +/- 0.05 (b)	"	"	0.79 (a)	0.20	p, in the presence of adenosine
Fig. 14	promethazine	1.47 +/- 0.04 (b)	"	"	1.05 (a)	0.18	deaminase. Similar to value
Fig. 15	doxepin	1.46 +/- 0.02 (b)	"	"	0.31 (a)	0.05	obtained in the absence of adenosine deaminase.
		1.45 +/- 0.01					(mean +/- SEM n = 4)

(Table 7. continued)

Shown is the stepwise fitting of the pharmacological data to equation 9 of the metactoid model. All curve fitting was performed on PROPHET using the FITFUN procedure (Haig and Reidmiller, 1980). Fitted dissociation constants (i.e. K_a, K_b, K_i, K_i') were obtained by fitting the pharmacological data of the Fig. No. shown to a modified version of equation 9 of the metactoid sensitization model:

$$\text{Percent Response to D} = \left[\frac{\alpha[D]}{[D] + K_a(1 + A/K_i)}\right] + \left[\frac{\gamma[D]}{[D] + K_b(1 + B/K_i')}\right] + \left[\frac{[D]}{[D] + K_a(1 + A/K_i)}\right]\cdot\left[\frac{\beta[D]}{[D] + K_b(1 + B/K_i')}\right]$$

Where:

α = percent of overall HA response due to direct stimulation at H_2-receptors (R_a, HA dissociation constant = K_a)

β = percent of overall HA response due to H_1-receptor stimulation (R_b, HA dissociation constant = K_b) dependent on concommitent stimulation of H_2-receptors.

γ = percent of overall HA response due to H_1-receptor stimulation dependent on endogenous adenosine for expression.

$$p = \frac{\alpha + \beta}{\alpha} \quad ; \quad r = \frac{\gamma \times p}{\alpha + \beta}$$

All other definitions as in equation 9 (see Results Ei)). Fitted values of p and r were obtained by fitting the Fig. No. shown directly to equation 9 of the metactoid model.

K*** = either K_i or K_i' in equation 9 of the metactoid model;
(i.e. K_i = dissociation constant of the competitive antagonist cimetidine presumed to act exclusively at H_2-receptors;
K_i' = dissociation constants of H_1-antagonists, presumed to act exclusively as competitive antagonists at H_1-receptors).

SS = the residual sum of squares of the fitted curve(s). Parameter values generating the lowest SS (for a given Fig. No) were assumed to reflect a better fit to the data.

(a) = Initial estimate;

(b) = Fitted value.

(c) = The fitted dissociation constants for K_a and K_b were determined by fitting a theoretical concentration response curve to HA (obtained by determining theoretical responses from the mean HA concentration-response curve parameters given in Table 6, for 21 HA data points evenly distributed over the concentration range .01 - 0.1 M.

* = Values used to obtain best estimate of K_b.

** = Values used to obtain best estimate if K_a.

2.62 uM, in the presence of adenosine deaminase the HA concentration-response curve was best fit when K_a was set to 1Ø uM with a fitted K_b of 2.43 uM (Table 7).

Additional curve fitting was performed on the control HA response in the presence and absence of HA-receptor antagonists. As noted above, varying K_a and fitting K_b revealed that, in absence of adenosine deaminase, the data were best fit by K_a = 6 uM with a fitted K_b of 2.67 +/- Ø.3Ø uM (Table 7).

The mean K_b of 2.67 uM obtained above was then used to refit the data to obtain a more accurate estimate of 5.6 uM for K_a (Table 7). These dissociation constants were used to determine the apparent dissociation constants of H_2- and H_1-antagonists.

The fitted dissociation constant for cimetidine (Ø.27 uM, see Table 7) is identical to our independent estimate (Ø.27 uM, see Fig. 7B) obtained in the absence of adenosine deaminase and EGTA. This value is also similar to the dissociation constant of cimetidine determined in the presence of adenosine deaminase and EGTA (Ø.18 uM, see Fig. 18).

The fit of cimetidine antagonism of the HA response in the absence of adenosine deaminase and EGTA is shown in Fig. 28. Note that the response to HA at higher concentrations of cimetidine (3 and 1Ø uM) fell to the

right of the fit to this data (Fig. 28). A similar deviation was observed when K_a was estimated at 10 uM (not shown).

In the absence of adenosine deaminase and EGTA, dissociation constants for H_1-antagonists were also obtained by fitting the data to the best estimates of K_a and K_b (Table 7). This fit suggested that if mepyramine and d-chlorpheniramine inhibited indirectly mediated H_1-receptor stimulation, their dissociation constants were in the nM range (Table 7). As expected, the fitted dissociation constant for l-chlorpheniramine was approximately 100 times greater than that for l-chlorpheniramine (Table 7).

The fit of the model to the mepyramine data is shown in Fig. 29A. Note that the theoretical curve for 0.01 uM mepyramine falls to the right of the data points. This latter observation also occurred when K_a was estimated at 10 uM (not shown).

In the absence of adenosine deaminase, the best fit dissociation constants obtained above were used to refit the HA concentration-response curves to obtain fitted values of p and r. As shown in Table 7, the fitted values of p and r were not significantly different from the initial estimates.

Fig. 28. Fit of cimetidine antagonism of the histamine response in the absence of adenosine deaminase to the metactoid model.

Shown is the best fit of cimetidine antagonism of HA-stimulated ^{3}H-cyclic AMP accumulation (data from Fig. 7A) to equation 9 of the metactoid model.

The fitted values are:

$p = 1.45$, $r = 0.36$, $K_a = 5.60$ uM, $K_b = 2.67$ uM,
K_i for cimetidine = 0.271 uM.

——— Curves were obtained by fitting data (0 (●), 1 (▲), 3 (■) and 10 (▼) uM cimetidine) to Equation 9 of the metactoid model.

----- Theoretical curve for cimetidine (300 uM (□)) inhibition of the HA response, derived from fitted values.

See Table 7 and text for further details.

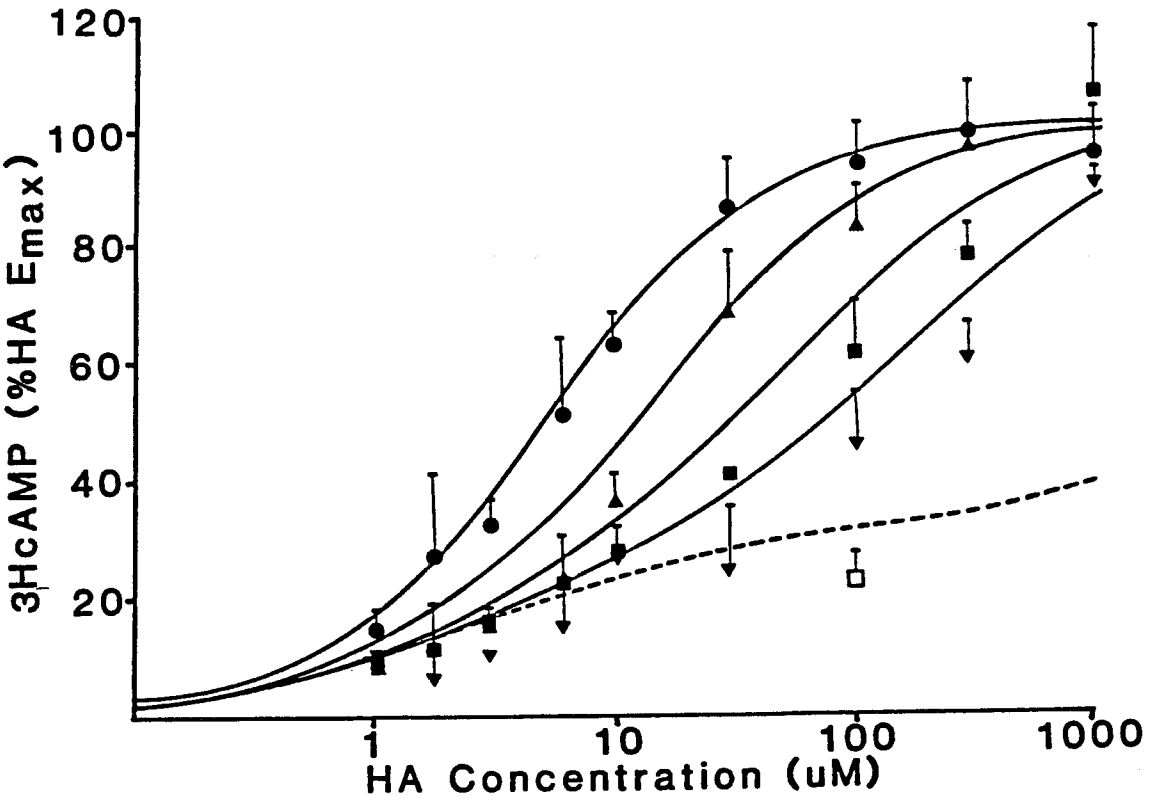

120
100
80
60
40
20
3HcAMP (%HA Emax)
1
10
100
1000
HA Concentration (uM)

Fig. 29. Fit of mepyramine antagonism of the histamine response in the presence and absence of adenosine deaminase to the metactoid model.

Shown are the best fits of mepyramine antagonism of the HA response in the absence [(A), data from Fig. 8A], or presence [(B), from combining the data of Figs. 14A, 14C and 17; n = 6, 2, 4 and 2 at mepyramine = Ø (●), Ø.Ø1 (□), Ø.1 (▼) and 1 (○) uM respectively] of adenosine deaminase to equation 9 of the metactoid model.

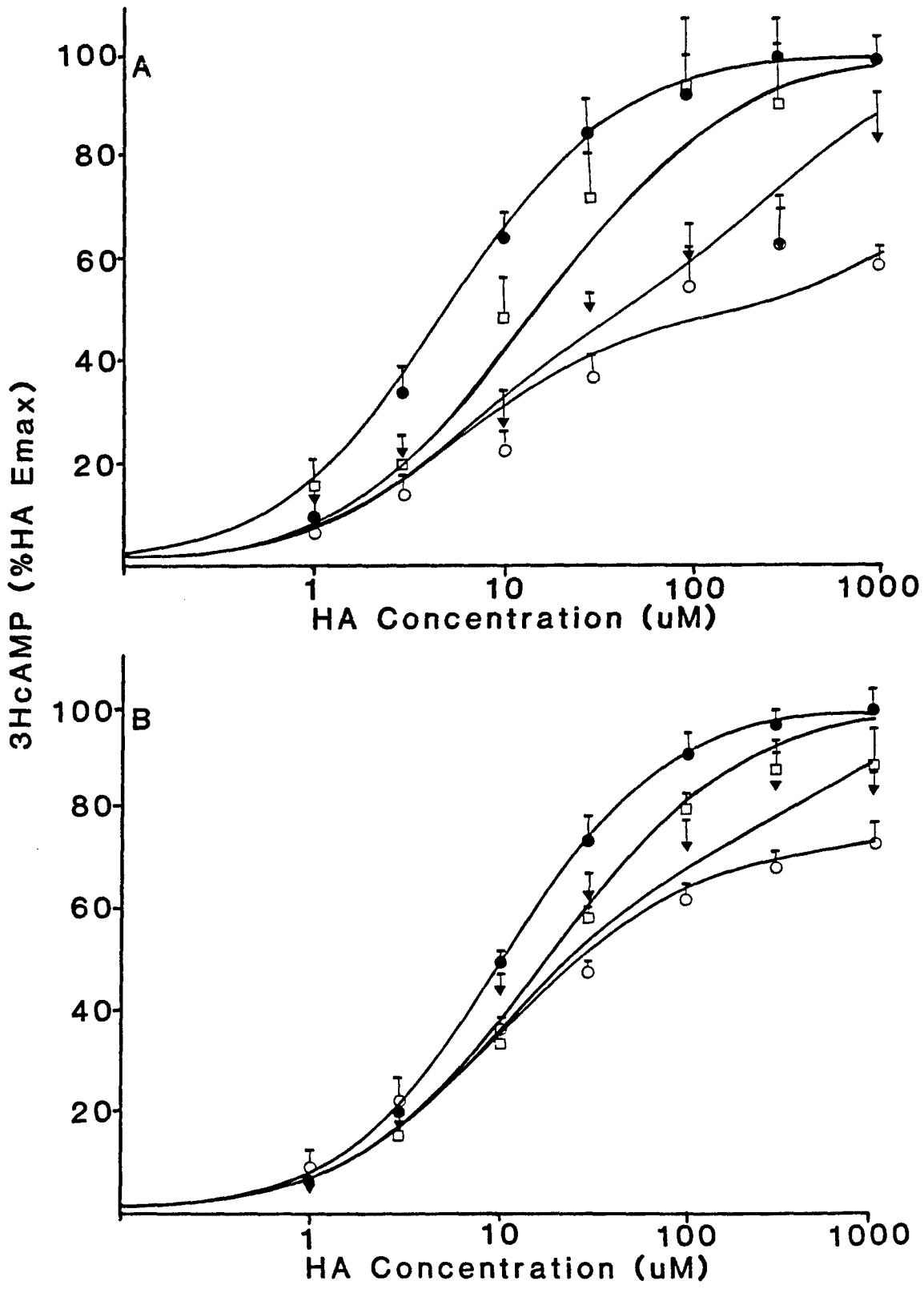
The fitted values are:

A. p = 1.45, r = Ø.36, K_a = 5.6Ø uM, K_b = 2.67 uM, K_i, for mepyramine = Ø.74 nM.

B. p = 1.45, r = Ø.ØØ, K_a = 1Ø.ØØ uM, K_b = 2.67 uM, K_i, for mepyramine = Ø.51 nM.

Both fits assume that mepyramine acts exclusively as a competitive antagonist at putative H_1-receptors.

See Table 7 and text for further details.


A
B
100
80
60
40
20
3HcAMP (%HA Emax)
1
10
100
1000
HA Concentration (uM)

Due to the small effect of H_1-antagonists on the HA response seen in the presence of adenosine deaminase (Fig. 14), attempts to obtain an accurate estimate of K_b in a fashion analogous to curve fitting procedures used in the evaluation of control responses (see Table 7) were unsuccessful. As found when the mean HA concentration-response (from Table 6 using theoretical data points, see Table 7 legend) was fit to the metactoid model, it was observed that the HA response (experimental data) in the absence of antagonists was better fit by K_a = 10 uM (not shown). However, when antagonist data was also included in the fitting procedure, the total residual sum of squares of the fitted curves were not significantly different when K_a was set to either 10 or 6 uM and K_b and apparent antagonist dissociation constants simultaneously fit to the metactoid model (Table 7).

In the presence of adenosine deaminase, since K_a and K_b estimates were more accurately reflected by the HA concentration-response in the absence of antagonists, estimates of antagonist affinities were obtained at K_a = 10 uM and K_b = 2.67 uM. This latter K_b value was selected on the basis of the similarity between the K_b of the HA response in the absence of adenosine deaminase and that derived when K_a was set to 10 uM in the presence of adenosine deaminase (Table 7). With these estimates of the HA dissociation constants, the fitted antagonist

dissociation constants for mepyramine and d-chlorpheniramine were similar to those obtained in the absence of adenosine deaminase (Table 7). Estimates of the dissociation constants of doxepin and promethazine were also obtained (Table 7).

The fit to mepyramine antagonism of the HA response in the presence of adenosine deaminase is shown in Fig. 29B. According to the metactoid model, the difference in the pattern of inhibition seen between Fig. 29A and 29B, is attributed to the presence (29A) or absence (29B) of the putative adenosine-dependent H_1-mediated component in the overall response to HA.

Fitted values of p were obtained by fitting HA concentration-response curves in the presence of adenosine deaminase using the best estimates of dissociation constants obtained under this incubation condition (see above). The best estimate of p obtained in the presence of adenosine deaminase was not significantly different from that obtained in the absence of adenosine deaminase (Table 7).

DISCUSSION.

I. Factors Influencing Basal and Histamine-Stimulated ^{3}H-Cyclic AMP Accumulation in the Vesicular Preparation.

Many factors influence the choice of tissue preparation and incubation conditions for studies of drug-induced changes in cyclic AMP synthesizing systems in brain. Parameters include consideration of tissue viability (in a specific brain preparation), buffer composition, pH and temperature. In pharmacological experiments, the dependent variable that yields information about drugs and drug receptors is the tissue response whilst the critical independent variable is the concentration of drugs at their receptor(s) (Kenakin, 1984). An advantage of the vesicular preparation for pharmacological studies is that drug concentrations at receptors are close to bath concentrations and can be controlled, unlike brain slice preparations. Similar advantages are apparent for the elucidation of biochemical control of brain responses.

This study documents some of the variables influencing both basal and HA-responsive changes in ^{3}H-cyclic AMP levels in the vesicular preparation. Further, evidence is presented suggesting how some of these variables can be controlled, leading to a preparation more suitable for

systematic studies of factors influencing neurohumoral-induced changes in ^{3}H-cyclic AMP levels.

The large flux of ^{3}H-cyclic AMP seen in the vesicular preparation when used with short preincubation times probably reflects a lack of steady-state endogenous cyclic AMP levels. Such short preincubation times (i.e. 15 min) were used by other investigators (e.g. Chasin et al., 1974; Daly et al., 1980; Psychoyos et al., 1982). A rapid increase in endogenous cyclic AMP occurred within 30 sec after animal sacrifice (Krishna et al., 1970), and in the initial incubation of hippocampal brain slice preparations (Whittingham et al., 1984). Extending preincubation times from 15 min to 40 or 60 min abolished the ^{3}H-cyclic AMP flux by assuring that endogenous cyclic AMP levels attain the steady state.

While ^{3}H-nucleotide formation was not influenced by preincubation time (Fig. 3), several reports suggest the presence of different ATP pools in brain (Skolnick and Daly, 1975; Shimizu and Okayama, 1973) and ganglia (Lindl et al., 1975). Specific activity changes in ^{3}H-cyclic AMP have been reported in response to HA and noradrenaline in brain slice studies (Schultz and Daly, 1973a; Chasin et al., 1973; Krishna et al., 1970) and noradrenaline in vesicular preparations (Chasin et al., 1974). This indicates that unlabeled endogenous ATP can also serve as a precursor for cyclic-AMP synthesis. Longer

preincubations times (prior to ^{3}H-adenine labeling) were also routinely used in an effort to preclude the possible deposition of ^{3}H-adenine to different ATP pools.

Several changes in the method were required in order to ensure adequate suspension of the vesicular preparation. Increased ^{3}H-cyclic AMP basal activity was noted when inadequately suspended preparations were mixed (Fig. 4). It was possible to alleviate this problem by use of large diameter vials which permit more adequate mixing. This observation is similar to that noted by Psychoyos et al., (1982). In this study, incubation in small diameter vessels, as used by others (Daly et al., 1980; McNeal et al, 1980), was associated with tissue sedimentation. Presently it was found that rapid mixing (necessary to prevent settling) caused deviations of 25 mM KRB buffer pH from around 7.4 to more alkaline values (Table 4). Use of supplementary buffers, or decreasing the bicarbonate concentration to 15 mM, maintained buffer pH at around 7.4.

The time-dependent decline in basal and HA responsive ^{3}H-cyclic AMP levels characteristic of bulk-labeled preparations may also be partially attributable to the flux of ^{3}H-nucleotides through specific nucleotide substrate pools (see above). My results confirm similar observations (McNeal et al., 1980) with bulk ^{3}H-adenine labeled vesicular preparations, in that both apparent

basal and absolute HA-stimulated ^{3}H-cyclic AMP levels decline as a function of total exposure time to ^{3}H-adenine (Fig. 5). This decline was associated with a decreased HA E_{max}, with no change in the HA EC_{50} (Fig. 5). McNeal et al (1980) suggested that the decline in basal and hormonally responsive ^{3}H-cyclic AMP can be attributed to continued exposure to low levels of extracellular adenosine, which decreased the ^{3}H-cyclic AMP response to other neurohormones through heterologous receptor desensitization. However, I found that prolonged vesicular preincubation prior to bulk ^{3}H-adenine labeling (with a constant ^{3}H-adenine labeling time) did not cause a decline in HA-stimulated ^{3}H-cyclic AMP levels (Fig. 5). This suggests that the decline in ^{3}H-cyclic AMP levels may not be caused by adenosine-mediated receptor desensitization. The observation (Newton and Hough, 1984 and results herein) that inclusion of adenosine deaminase throughout all incubation stages, in an individually labeled vesicular preparation, caused an approximate 25% increase in ^{3}H-nucleotide levels also imputes adenosine. One mechanism to explain the decline in hormonally-responsive ^{3}H-cyclic AMP seen in bulk-labeled vesicular preparations might be that prolonged exposure of adenosine during incubation of the vesicular preparation following ^{3}H-adenine labeling could result in displacement of ^{3}H-ATP from specific substrate pools linked to ^{3}H-cyclic AMP accumulation.

The decline in the HA response seen in bulk-labeled vesicular preparations, makes this preparation unsuitable for pharmacological analysis. In particular, in several instances high concentrations of HA (300 uM) gave lower ^{3}H-cyclic AMP levels than low concentrations (10 uM) added less than 1 min earlier. A more stable vesicular preparation was obtained by maintaining a constant ^{3}H-adenine labeling time by individual labeling of vesicular aliquots with ^{3}H-adenine. Under these conditions basal and HA responsiveness were maintained over a 14 min period (Fig. 5). This stabilization of the HA response was accompanied by a decrease in HA fold stimulation, relative to bulk-labeled preparations (Fig. 6), perhaps as a consequence of decreased oxygen delivery to the tissue. Nonetheless, the degree of HA-stimulation in individually-labeled preparations was in the same range as other reported values determined under bulk-labeling conditions (Chasin et al., 1974; Daly et al., 1980).

My findings indicate that basal ^{3}H-cyclic AMP levels are not an absolute predictor of neurohumorally responsive ^{3}H-cyclic AMP accumulation. Adequate vesicular mixing resulted in a lower apparent ^{3}H-cyclic AMP basal activity, and was associated with high neurohumoral-induced ^{3}H-cyclic AMP accumulation (Psychoyos et al., 1982), leading to the hypothesis that low basal activity increased neurohumoral responsiveness. Likewise, I have

demonstrated that high ^{3}H-cyclic AMP basal activity in bulk-labeled phosphate preparations is associated with a lower fold HA-stimulation. However, for a given incubation condition, this hypothesis is not validated. Absolute HA-responsiveness was higher with increasing basal activity, an effect clearly not paralleled by an increased fold HA-stimulation. Comparisons among different incubation conditions also demonstrated that low basal activity was not associated with higher HA-responsiveness (Fig. 6).

Part of the differences in apparent basal, absolute and fold HA-stimulation noted in different vesicular preparations may be attributable to the presence of endogenous adenosine in the incubation medium. Extracellular adenosine, derived from the vesicular preparation, appears to contribute to apparent basal activity and also influences ^{3}H-cyclic AMP responses to some neurohumoral agents. Inclusion of adenosine deaminase significantly decreased both apparent basal activity and absolute HA-responsiveness (McNeal et al., 1980; Newton and Hough, 1984). These changes have been suggested to reflect elimination of putative indirectly acting H_1-mediated adenosine-dependent ^{3}H-cyclic AMP accumulation (Daly et al. 1980), a hypothesis supported by this study (see Discussion 2.). I have previously demonstrated (Newton and Hough, 1984) that prolonged

incubation with adenosine deaminase decreased basal activity more than HA-responsiveness, resulting in an improved HA-fold stimulation. Thus, other factors notwithstanding, the relationships between endogenous adenosine contributions to basal ^{3}H-cyclic-AMP activity, and the hormonal responses seen require further study.

In addition to differences in basal activity and maximal HA responsiveness encountered with different incubation conditions, differences were also noted in HA-concentration response curves. The EC_{50} of HA concentration response curves, which ranged from 3-20 uM in all preparations (Table 5), compares favorably with similar studies in cortical vesicular preparations (EC_{50} = 20 uM, Chasin et al., 1974; EC_{50} = 10 uM, Psychoyos, 1981) and cortical (EC_{50} = 7 uM, Baudry et al., 1975) or hippocampal brain slice studies (EC_{50} = 12 uM, Dismukes et al., 1976b). The EC_{50} of HA response curves following individual labeling in 15 mM KRB was significantly lower than the EC_{50} obtained after incubation in phosphate or hepes (Table 5). Because individual labeling and incubation in 15 mM KRB gave a stable, reproducible response to HA, this incubation condition was utilized in the current study to characterize HA-receptor(s) mediating ^{3}H-cyclic AMP accumulation in the vesicular preparation.

The differences in HA-responsiveness documented above reflect as yet little understood changes in the efficiency of HA-receptor(s) coupled to ^{3}H-cyclic AMP accumulation in the vesicular preparation. I conclude that, at least for the case of HA, individual labeling of vesicular aliquots represents a significant improvement in methodology which allows for greater control over the biochemical and pharmacological analysis of mechanisms controlling cyclic AMP dynamics in brain. This preparation might also prove useful in the study of other hormonal and biochemical factors influencing cyclic AMP dynamics in brain.

2. Metactoid Sensitization as a Model for Histamine Stimulated ^{3}H-Cyclic AMP Accumulation in the Cortical Vesicular Preparation.

2.1. Evidence for H_1- and H_2-Receptor Involvement in the ^{3}H-Cyclic AMP Response to Histamine.

A significant H_2-receptor component in the ^{3}H-cyclic AMP response to HA was revealed by the concentration-dependent inhibition of this response by the H_2-antagonist cimetidine (Fig. 7). The concentration-response to the H_2-agonist dimaprit was also antagonized by cimetidine (3 uM) (Fig. 11) confirming an H_2-receptor coupled to ^{3}H-cyclic AMP accumulation in this preparation. However, the HA response could not be entirely attributed to direct H_2-receptor stimulation. A cimetidine-resistant component accounting for about 20% of the maximum response to HA was observed (Fig. 7 and 13). Cimetidine acted as a competitive antagonist of the remaining 80% of the HA response (Fig. 7B). The derived apparent dissociation constant (K_{iapp}) for cimetidine in inhibiting this component of the HA response (0.27 uM) or in inhibiting dimaprit-stimulated ^{3}H-cyclic AMP accumulation (0.49 uM) was similar. These cimetidine K_{iapp} values are also similar to those obtained from other H_2-mediated processes, such as contraction of guinea pig atria (Black et al., 1972), and strongly support the hypothesis that H_2-receptors mediate a major portion of HA-stimulated

^{3}H-cyclic AMP accumulation in the vesicular preparation.

An H_1-receptor involvement in the ^{3}H-cyclic AMP response to HA also seems likely. The H_1-antagonists mepyramine, d- and l-chlorpheniramine antagonized the HA response in a concentration-dependent fashion (Fig. 8). Mepyramine and d-chlorpheniramine were approximately equipotent in inhibiting the HA response (Fig. 8). Importantly, the l-isomer of chlorpheniramine was approximately 100 times less potent than its stereoisomer, in agreement with the difference in the ability of these agents to inhibit ^{3}H-mepyramine binding or other H_1-mediated processes (see Table 1).

In contrast to the large proportion of H_2-receptor involvement in the response to HA (80%), only 45% of the overall response to HA appears to be mediated by H_1-receptor stimulation, and the remaining 55% of the ^{3}H-cyclic AMP response to HA (100 uM) was not blocked by these agents at concentrations up to 100 uM (Fig. 9). This 55% was probably mediated only through H_2-receptor stimulation, since a combination of H_1- and H_2-antagonists was required to completely block the HA response (100 uM) (Fig. 13). Since other studies suggested that 80% of the HA response was mediated by H_2-receptor stimulation (see above), it follows that total inhibition by H_1- plus H_2-antagonists equals 125%. Therefore 25% of the HA response sensitive to inhibition by H_1-antagonists was also

sensitive to inhibition by H_2-antagonists (see Fig. 23).

Further experimentation suggested that H_1-antagonists did not inhibit the HA response through an action at H_2-receptors. The concentration-response to dimaprit was not antagonized by mepyramine (Ø.1 uM) (Fig. 12). In a similar vesicular preparation of guinea pig cortex, others have shown that ^{3}H-cyclic AMP accumulation formed in response to the selective H_2-agonist 4-methyl-HA (1ØØ uM) was not antagonized by the H_1-antagonist tripelennamine (Ø.3 - 1Ø uM) (Psychoyos, 1978). Thus these concentrations of H_1-antagonists (Ø.1 - 1Ø uM) do not block H_2-mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation.

Taken together it can be argued that 25% of the response to H_1-receptor stimulation may require concommitant activation of H_2-receptors for expression. Consistent with this hypothesis, in a similar preparation of guinea pig cerebral cortex, Psychoyos (1978) demonstrated that at least 70% of the ^{3}H-cyclic AMP response to 2-methyl-HA (1ØØ uM) (a selective H_1-agonist which would be predicted to occupy both H_1- and H_2-receptors at this concentration) was inhibited by the H_2-antagonist metiamide (1Ø uM) and up to 8Ø% was inhibited by the H_1-antagonist tripelennamine (Ø.1 - 3 uM). At the lowest concentration tested (Ø.1 uM), tripelennamine inhibited 4Ø% of the response to 2-methyl-HA (1ØØ uM).

Assuming tripelennamine acted specifically to antagonize the response to 2-methyl-HA at H_1-receptors, these results imply that some fraction of the response to 2-methyl-HA required activation of both H_1- and H_2-receptors.

About 20% of the HA response appears to reflect an H_1-component stimulating ^{3}H-cyclic AMP accumulation independent of H_2-receptor stimulation. Cimetidine only blocked 80% of the HA response and a combination of H_1- and H_2-receptor antagonists was required to abolish the HA response (Fig. 13). As discussed below, this H_1-component was abolished by adenosine deaminase, which suggested that all H_1-mediated ^{3}H-cyclic AMP accumulation was dependent on either prior H_2-receptor stimulation or adenosine.

The pattern of inhibition of the HA response seen in the presence of H_1- and H_2-receptor antagonists is not that expected if H_1- and H_2-receptors were independently coupled to ^{3}H-cyclic AMP accumulation in the vesicular preparation (Ariens et al., 1956). According to this model, H_1- or H_2-antagonists would inhibit only that portion of the HA response attributable to direct stimulation of H_1- or H_2-receptors, respectively. This would be observed as concentration-dependent surmountable shifts in that portion of the HA response mediated by a given receptor-subtype. Thus, increasing concentrations of selective H_2-antagonists, by blocking H_2-receptors, should reveal the extent of directly mediated H_1-receptor

involvement. Similarly, maximum blockade of H_1-receptors should reveal the extent of H_2-involvement in the HA response, with the two components together accounting for 100% of the response. In the present study, both H_1- and H_2-antagonists caused concentration-dependent surmountable antagonism of part of the HA concentration-response curve (Fig. 7 and 8). However, maximum blockade of the HA response with these antagonists (Fig. 7 and 9) revealed that these two components accounted for 125% of the HA response, an observation clearly incompatible with the independent 2 site model.

The pharmacological characteristics of the HA response can be accounted for by a metactoid model, in which H_2- and adenosine-receptor stimulation directly increase ^3H-cyclic AMP accumulation and H_1-receptor stimulation indirectly potentiates the response to either of these direct stimuli. Theoretical simulations (Figs. 25 - 27) of the metactoid model revealed that, if the HA response was mediated through an analogous mechanism, the dissociation constants of HA for H_1- and H_2-receptors (presently assumed to equal HA EC_{50} values at these receptors) must be similar to each other. This relationship was predicted from two observations. Namely, if only H_2-receptor stimulation permits expression of H_1-receptor involvement in the HA response, the overall EC_{50} of the response to HA cannot be less than its EC_{50} at H_2-

receptors (Fig. 25). If H_1-receptors can also potentiate the ^{3}H-cyclic AMP response due to adenosine-receptor stimulation, the HA EC_{50} at H_1-receptors cannot be considerably less than the HA EC_{50} at H_2-receptors since this would have been observed as a biphasic concentration-response to HA (Fig. 27B), which was not seen.

The best fit of the pharmacological characteristics of the HA response to the metactoid model generated EC_{50} estimates of 2.67 and 5.6 uM for HA at H_1- and H_2-receptors, respectively (Table 7). This estimate of the EC_{50} at H_1-receptors was similar to the estimated EC_{50} of the cimetidine-resistant component of the HA response (Fig. 7A). The K_{iapp} of cimetidine (Ø.271 uM) obtained from fitting the HA concentration-response (Fig. 28) to these EC_{50} estimates was identical to previously obtained estimates (from Fig. 7B) and similar to those derived from other H_2-mediated processes (Table 2 and Black et al., 1972). Similarly, the dissociation constants for H_1-antagonists on this response resemble those derived from conventional H_1-mediated responses, such as contraction of the guinea pig ileum, and from binding studies (cf. Table 7 vs Table 1). Thus, the observed pharmacological characteristics of the HA response are adequately described by the metactoid sensitization model.

2.2. Evidence for Adenosine Involvement in the ^{3}H-Cyclic AMP Response to Histamine.

Several findings suggested that adenosine may potentiate the cyclic AMP response to H_1-receptor stimulation in brain slice and vesicular preparations (see Introduction 6.3.3.). In particular, McNeal et al., 1980, demonstrated that inclusion of adenosine deaminase (10 ug/ml) caused a decrease in both basal and HA-responsive ^{3}H-cyclic AMP accumulation in a ^{3}H-adenine prelabeled vesicular preparation of guinea pig cortex. This raised the possibility that an H_1-component dependent on endogenous adenosine was contributing to the ^{3}H-cyclic AMP response to HA seen in the present study.

Inclusion of adenosine deaminase (2.5 U/ml) caused a large decrease in ^{3}H-cyclic AMP basal activity (Table 6), suggesting that extracellular adenosine concentrations were sufficient to activate adenosine-receptors coupled to ^{3}H-cyclic AMP accumulation in this vesicular preparation. This reduction in ^{3}H-cyclic AMP basal activity was accompanied by a reduction in the sensitivity of the HA response to inhibition by H_1-antagonists (cf. Fig. 8 vs 14) and a shift in the overall HA EC_{50} from 5.91 to 11.36 uM (Table 6). In the presence of adenosine deaminase, the maximal inhibition of the HA response by H_1-antagonists was about 30% compared to 45% in the absence of this enzyme (Figs. 9 vs 14). This suggests that, in the

absence of adenosine deaminase, adenosine may have potentiated the ^{3}H-cyclic AMP response to H_1-receptor stimulation.

The hypothesis that part of the HA response is mediated by an adenosine-H_1-receptor interaction is supported by the observation that the entire HA response (100 uM) was inhibited by cimetidine (300 uM) in the presence of adenosine deaminase, while in its absence 20% of the HA response was cimetidine-insensitive (Fig. 13). Thus, in the presence of adenosine deaminase, it appears that H_1-receptor mediated ^{3}H-cyclic AMP accumulation is entirely dependent on concomitant H_2-receptor stimulation. Interestingly, the fraction of the H_1-response dependent on H_2-receptor stimulation did not appear to change with inclusion of adenosine deaminase (Fig. 23). The hypothesis that adenosine potentiates H_1-mediated ^{3}H-cyclic AMP accumulation through stimulation of adenosine receptors (as opposed to other actions of this nucleoside and/or adenosine deaminase) requires alternative confirmation (e.g. by use of adenosine receptor antagonists).

The small effects of H_1-antagonists on the HA response seen in the presence of adenosine deaminase were confirmed to occur through antagonism of the HA response at H_1-receptors. Thus, in the presence of adenosine deaminase, as in its absence, the response to dimaprit (100 uM) was

not antagonized by mepyramine (Ø.1 uM) (Fig. 22). In addition, in the presence of adenosine deaminase, a series of structurally diverse H_1-antagonists inhibited the HA response at concentrations selective for H_1-blockade (see Fig. 14 and Table 1 vs 2). The rank order of potency of these agents i.e. mepyramine = promethazine = d-chlorpheniramine > diphenhydramine ≥ l-chlorpheniramine (Fig. 14) was that predicted from other H_1-mediated responses (see Table 1).

It is unclear whether the tricyclic antidepressants doxepin and amitryptyline also inhibited the HA response through competitive antagonism at H_1-receptors. According to their K_{bapp} values determined from inhibiting ^3H-mepyramine to rat brain (see Introduction 4.1.), doxepin (1 nM) or amitryptyline (1Ø nM) would be expected to cause a greater inhibition of the ^3H-cyclic AMP response to HA than mepyramine (1Ø nM), which did not appear to occur in the current study (Fig. 15). This anomaly did not appear to be due to an overestimation of free drug concentration, as suggested to occur in some binding studies (see Introduction 4.1.), since doxepin (1 nM) also failed to inhibit the ^3H-cyclic AMP response to HA when protein content was decreased. The effect of these antidepressants on H_1-mediated ^3H-cyclic AMP accumulation may be obscured by other effects of these drugs e.g. concomitant inhibition of phosphodiesterase (Levin and Weiss, 1976).

In the presence of adenosine deaminase, the best estimates of HA EC_{50} values at H_1- and H_2-receptors obtained from fitting the pharmacological data to the metactoid sensitization model were 2.67 and 10 uM, respectively (Table 7). With these estimates, the dissociation constants of H_1-antagonists obtained by fitting the pharmacological data to the metactoid sensitization model (Table 7) were in good agreement with those obtained in the absence of adenosine deaminase (Table 7) and other H_1-mediated processes (Table 1). Note however, that within the context of the metactoid model, best fit estimates of the HA EC_{50} at H_2-receptors should have been identical in the presence and absence of adenosine deaminase, which was not the case (10 vs 5.6 uM, respectively; Table 7). This does not imply that metactoid sensitization does not account for the mechanism whereby H_1-receptors stimulate ^{3}H-cyclic AMP accumulation in the vesicular preparation. Rather, this observation questions the validity of the assumptions on which the metactoid model was based. This discrepancy is discussed elsewhere (see Discussion 2.4.).

2.3. Evidence for Calcium Involvement in the ^{3}H-Cyclic AMP Response to Histamine.

It was of interest to determine whether H_1-mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation was calcium-dependent, as has been suggested for H_1-receptors

coupled to cyclic AMP accumulation in the guinea pig cortical brain slice (Schwartz et al., 1980a). Addition of EGTA (2 mM) caused a large increase in ^{3}H-cyclic AMP basal activity, which appeared to mask the response to HA (100 uM) and was therefore not investigated further (not shown). In the presence of EGTA (2 mM) and adenosine deaminase (2.5 U/ml), the H_1-antagonist mepyramine (0.1 uM) had no effect on the HA response (Fig. 17). This infers that the H_1-component noted in either the presence or absence of adenosine deaminase was abolished by the addition of EGTA and might therefore be calcium-dependent. However, since calcium-removal probably causes changes in membrane structure, and EGTA (2 mM) may have additional effects unrelated to calcium chelation, the hypothesis that H_1-mediated ^{3}H-cyclic AMP accumulation is calcium-dependent requires additional investigation.

The loss of the H_1-component in the presence of adenosine deaminase and EGTA was supported by the observation that only H_2-receptors mediated ^{3}H-cyclic AMP accumlation under these conditions. Thus, in the presence of EGTA and adenosine deaminase, the H_2-antagonists cimetidine (Fig. 18) and metiamide (Fig. 19) both acted as classical competitive antagonists of the ^{3}H-cyclic AMP response to HA. The similarity of K_{iapp} values for the metiamide (0.71 uM) and cimetidine (0.18 uM) in inhibiting

HA-induced increases in ^{3}H-cyclic AMP under these incubation conditions, or in inhibiting other H_2-mediated processes (see Table 1 and Black et al., 1972) argues that H_2-receptor occupancy determines all these HA responses.

In contrast to cimetidine and metiamide, the H_2-antagonist tiotidine, while acting at concentrations expected for H_2-receptor involvement (Ø.Ø1 - 3Ø uM), inhibited the HA response noncompetitively in the vesicular preparation (Fig. 2Ø). This observation is in contrast to that obtained in broken cell preparations where tiotidine competitively inhibited H_2-activated adenylate cyclase in broken cell mebranes of guinea pig brain (Maayani et al., 1982). In the present study, tiotidine was prequilibrated for 2Ø min prior to the addition of HA. This raises the possibility that, in the vesicular preparation, tiotidine acts pseudoirreversibly at H_2-receptors, perhaps because this preincubation time is longer than has been used previously by others. It would be informative to characterize the effects of other H_2-antagonists, particularly nonimidazole derivatives like tiotidine, on H_2-mediated cyclic AMP accumulation.

The absolute magnitude of H_2-receptor mediated ^{3}H-cyclic AMP accumulation does not appear to be significantly altered by adenosine deaminase, with or without EGTA. While adenosine deaminase and EGTA caused a 5Ø% reduction in the HA E_{max} compared to incubation in the

absence of these agents (Table 6) this appears largely attributable to removal of the H_1-component in the HA response. The magnitude of directly mediated H_2-stimulated ^{3}H-cyclic AMP accumulation (i.e. that fraction of the HA response not blocked by H_1-antagonists) seems similar in the absence or presence of adenosine deaminase with or without EGTA (Fig. 23). In addition, the ^{3}H-cyclic AMP response to dimaprit (100 uM) was unchanged by these treatments (Fig. 22). This reduction in the maximum HA response therefore appears to represent a selective loss of the H_1-component.

Additional evidence that an H_1-component was eliminated by adenosine deaminase and EGTA was obtained from agonist studies. Compared to incubation in the absence of these agents, the concentration-response to the mixed HA-receptor agonists HA and TEA were shifted to the right by this treatment, while the response to the specific H_2-agonist dimaprit was not altered (Fig. 21 and Table 6). Since TEA is a selective H_1-agonist and the EC_{50} for this agonist at H_1- and H_2-receptors are at least an order of magnitude apart (Green and Hough, 1980), the shift to the right in the concentration-response to this agonist also supports the hypothesis that an H_1-component, independent of H_2-receptor stimulation, occurs in the absence of EGTA and adenosine deaminase.

Interestingly, the EC_{50} of the HA responses were similar in the presence of adenosine deaminase with (16.22 uM) or without (11.36 uM) EGTA (Table 6). This observation is in general agreement with the predictions of the metactoid sensitization model, which infers that if H_1-mediated ^3H-cyclic AMP accumulation is dependent on H_2-receptor stimulation (as seen in the presence of adenosine deaminase alone) the EC_{50} of this response cannot be less than the EC_{50} at H_2-receptors (assumed to be reflected by the EC_{50} of the HA response in the presence of adenosine deaminase and EGTA).

2.4. Alternative Assumptions in the Metactoid Sensitization Model.

HA-mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation can be largely accounted for by the metactoid sensitization model (see Discussion 2.1 - 2.3.). However, the observation that the HA EC_{50} was shifted to the right by adenosine deaminase (Table 6) was not predicted by the metactoid sensitization model. Given the occupancy assumptions of the metactoid model, in the presence of adenosine deaminase, the overall EC_{50} of the HA concentration-response cannot be less than the dissociation constant at H_2-receptors, regardless of the HA dissociation constant at H_1-receptors (see Fig. 25). According to the predictions of the model, when the dissociation constant at H_1-receptors is less than at H_2-receptors, the H_1-component would only be revealed as a shift to the left in the HA concentration-response (relative to the dissociation constant at H_2-receptors) when a large proportion of the HA response occurred through an H_1-component independent of H_2-receptor stimulation. This situation did not occur in the present study. In the absence of adenosine deaminase, only 20% of the total HA response could be accounted for as an H_1-response occurring independently of H_2-receptor stimulation (see Fig. 23). These observations suggests that one or more of the assumptions of the metactoid model

might require modification.

One explanation for the adenosine deaminase induced change in the HA EC_{50} might be to assume that stimulation of adenosine- and H_2-receptors does not result in ^3H-cyclic AMP accumulation that is additive. Perhaps adenosine and H_2-receptor stimulation exhibit positive cooperativity in the generation of ^3H-cyclic AMP. Thus, the HA EC_{50} at H_2-receptors would be lower in the presence than in the absence of adenosine. This hypothesis might be addressed by studying the effects of combinations of adenosine and HA in the presence of H_1-antagonists to exclude H_1-potentiation of this response.

Other non-receptor actions of adenosine may also alter the ^3H-cyclic AMP response to HA-receptor(s) stimulation. For example, incorporation of unlabeled adenosine into ATP might decrease the specific activity of ^3H-ATP linked to HA-mediated ^3H-cyclic AMP accumulation. In this study, inclusion of adenosine deaminase caused a 10% increase in ^3H-nucleotide levels (not shown). While inclusion of this enzyme did not appear to influence the maximum response to H_2-receptor stimulation (Fig. 23), the possibility that changes in the distribution of endogenous nucleotides altered the EC_{50} of the HA response has not been excluded. In this respect, use of an adenosine-receptor antagonist as an alternative method of eliminating the presumed adenosine-receptor dependent H_1-component of the HA

response would prove enlightening.

The metactoid model presented assumes a direct linear response relationship between all reacting components (i.e. all stimuli are directly related to receptor occupancy). However, there is evidence suggesting that in many biological responses the relationship between receptor occupancy and tissue response is nonlinear, i.e. the maximum response is obtained prior to full occupancy of the receptors (see Kenakin, 1984). If one step in the chain of events following a given receptor stimulus initiating increased ^{3}H-cyclic AMP levels reaches saturation prior to full occupancy of that receptor, then a spare capacity exists i.e. a receptor reserve. How the presence of spare receptors for either direct or metactoid stimuli might influence the characteristics of HA-mediated ^{3}H-cyclic AMP accumulation in the present study is unclear and awaits further definition of the effector mechanisms mediating the responses to H_1- and H_2-receptors.

3. Relationship of Histamine Receptors Subserving ^{3}H-Cyclic AMP Accumulation in the Vesicular Preparation to Other In Vitro Preparations of Brain.

The characteristics of H_1- and H_2-receptors mediating ^{3}H-cyclic AMP accumulation seen in the present study on a guinea pig cortical vesicular preparation are similar to those observed in brain slice studies (see Introduction 3.3). In contrast, only the H_2-receptor appears to be coupled to adenylate cyclase activation in broken cell membrane preparations (see Introduction 3.3.). Consideration of the similarities and differences between HA-receptors mediating cyclic AMP accumulation in these three brain preparations is given below.

The H_2-receptor seems to mediate cyclic AMP accumulation in preparations of broken cell membranes, brain slices or vesicles. Cimetidine and metiamide act as competitive antagonists at H_2-receptors in all these brain preparations (see Table 2, Fig. 18 and 19). Derived pA_2 values for both these antagonists are similar in the three brain preparations (see Table 2 vs Fig. 18 and 19). Since cimetidine and metiamide act as competitive antagonists on many H_2-mediated processes, including increased guinea pig atrial rate and relaxation of rat uterus (Black et al., 1972), an H_2-receptor presumably mediates all these HA responses.

Differences are also apparent in the pharmacological characteristics of H_2-receptors mediating cyclic AMP accumulation in different brain preparations. In brain slices, high concentrations (e.g. 100 uM) of H_1-antagonists inhibit presumed H_2-receptor mediated cyclic AMP accumulation, but in broken cell membrane preparations lower (e.g. 10 uM) concentrations are required to block H_2-mediated adenylate cyclase activation (see Table 2 and Trung Tuong et al., 1980). Mepyramine (100 uM) also had no effect on H_2-mediated ^{3}H-cyclic AMP accumulation (formed following ^{3}H-adenine prelabeling) in dissociated cells from guinea pig hippocampus (Kanba and Richelson, 1983). Similarly, based on its apparent affinity in inhibiting H_2-mediated adenylate cyclase activity in broken cells, promethazine (1 uM) would be predicted to inhibit H_2-mediated ^{3}H-cyclic AMP accumulation in the vesicular preparation about 75%. This was not seen in the present study. In the presence of adenosine deaminase, promethazine (1 uM) caused about a 30% inhibition of the HA response (Fig. 14) which was attributed to the H_1-antagonist properties of this drug. The increased ability of H_1-antagonists to inhibit presumed H_2-mediated cyclic AMP accumulation in broken cell membranes might be attributable to exposure of sites of action for these agents which are not accessible in more intact preparations of brain (e.g. the internal face of the plasma membrane). Alternatively, the H_2-receptor and/or

transduction mechanisms coupled to cyclic AMP accumulation could be modified in broken cells relative to brain slice or vesicular preparations.

It may be pertinent that the effects of H_1-antagonists on H_2-mediated cyclic AMP accumulation in brain slice and vesicular preparations were obtained in the presence of extracellular calcium while those in broken cell membrane preparations were determined in the presence of EGTA (which binds free calcium). The possibility that in the presence of EGTA, H_1-antagonists exert similar inhibitory effects on H_2-mediated cyclic AMP accumulation in these three brain preparations has not been excluded. It should be noted however, that the H_2-antagonist tiotidine did not act as a competitive antagonist of H_2-mediated ^3H-cyclic AMP accumulation in the vesicular preparation (Fig. 2Ø) in the presence of EGTA and adenosine deaminase, although this agent competitively antagonized H_2-mediated adenylate cyclase activation in broken cell preparations of guinea pig cortex (Maayani et al., 1982).

As previously noted, in the presence of EGTA and adenosine deaminase, only H_2-receptors are coupled to ^3H-cyclic AMP accumulation in the vesicular preparation. Under these conditions, the magnitude of maximum H_2-stimulated ^3H-cyclic AMP accumulation (2.68 times ^3H-cyclic AMP basal activity, Table 6) is larger than H_2-stimulated adenylate cyclase activation in broken cell

membrane preparations (up to a doubling of basal activity, Maayani, 1982). This observation may reflect an improved maintenance of H_2-mediated adenylate cyclase transduction mechanisms in the vesicular preparation. Alternatively, other, as yet unidentified H_2-mediated processes may be present in the vesicular preparation which are lost and/or changed in the broken cell membrane preparation. This latter hypothesis might account for the different pharmacological properties of HA-receptor antagonists observed between these preparations.

The relationship between the magnitude of H_2-receptor involvement in HA-mediated cyclic AMP accumulation in brain slices compared to vesicular preparations is unknown. Differences in the extent of H_2-receptor involvement in HA-stimulated cyclic AMP accumulation in guinea pig cerebral cortical slices have been reported (see Introduction 3.3.3.). My study suggests that the HA response observed in the presence of adenosine deaminase and EGTA is directly attributed to H_2-receptor stimulation, while in one study in brain slices this combination of agents eliminated the cyclic AMP response to HA (Schwabe et al., 1978).

The metactoid nature of H_1-mediated ^{3}H-cyclic AMP accumulation seen in the present study is also likely to function in HA-mediated cyclic AMP accumulation in brain slices. In both guinea pig hippocampal and rabbit

cortical slice preparations, in the absence of exogenous adenosine, H_2-antagonists caused a concentration-dependent shift to the right of the entire cyclic AMP response to HA (Palacios et al., 1978a; Al-Gadi and Hill, 1984). In contrast, H_1-antagonists inhibited only about 50% of the HA response and caused a surmountable shift to the right of the upper part of the HA concentration-response curve in both these slice preparations (Palacios et al., 1978a; Al-Gadi and Hill, 1984). This pattern of inhibition is similar to that observed in the present study, where, in the presence of adenosine deaminase, H_1-mediated ^{3}H-cyclic AMP accumulation appeared completely dependent on H_2-receptor activation (Fig. 13) and H_1-antagonists caused concentration-dependent surmountable shifts in the upper part of the HA concentration-response curve (Fig. 14).

The observation that endogenous adenosine potentiates the ^{3}H-cyclic AMP response to HA in the vesicular preparation is also paralleled by similar observations in brain slices. In guinea pig cortical (Daum et al., 1982; Hill et al., 1981a) and hippocampal (Dismukes et al., 1976b), or rabbit cortical (Al-Gadi and Hill, 1984) slice preparations, addition of exogenous adenosine (0.1 mM) increased the cyclic AMP response to HA or HA agonists. This increase appeared sensitive to inhibition by H_1-antagonists (Dismukes et el., 1976b; Daum et al., 1982; Hill et al., 1981a; Al-Gadi and Hill, 1984). Dissociation

constants of H_1-antagonists in inhibiting the HA-potentiation of the response to adenosine (100 uM) were similar to those expected if H_1-receptors mediated this HA response (see Table 1 and Hill et al., 1981a). In guinea pig hippocampal slices, the ^{14}C-cyclic AMP response to 4-methyl-HA (100 uM) or TEA (100 uM) is increased by inclusion of adenosine (100 uM) (Dismukes et al., 1976b). In this study, the response to TEA was antagonized by both d-brompheniramine (10 uM) and metiamide (10 uM) in the absence of exogenous adenosine, while in its presence only d-brompheniramine was an effective antagonist. In contrast, the response to 4-methyl-HA was antagonized only by metiamide in the absence of adenosine, while in its presence both metiamide and d-brompheniramine were effective antagonists. These results all suggest that H_1-receptor stimulation potentiates the cyclic AMP response to exogenous adenosine in brain slice preparations.

Interestingly, addition of exogenous adenosine, while increasing the magnitude of the HA response and its sensitivity to inhibition by H_1-antagonists, did not alter the HA EC_{50} (12 uM) in guinea pig cortical brain slices (Dismukes et al., 1976b). This might suggest that, as in the present study (Table 7), the HA EC_{50} at H_1- and H_2-receptors is similar in guinea pig cortical slices.

Endogenous adenosine may also have influenced the cyclic AMP response to HA in a guinea pig cortical slice

preparation, since adenosine deaminase (1.6 U/ml) reduced both basal and HA-stimulated (100 uM) cyclic AMP accumulation (Hill et al., 1981a). In this study, H_2-receptor stimulation did not appear to be involved in the HA response since cimetidine (100 uM) did not antagonize the response to HA (100 uM) and dimaprit (1 mM) had no significant effect on cyclic AMP accumulation. This therefore suggests that the decrease in HA responsive cyclic AMP accumulation seen in the presence of adenosine deaminase was due to loss of an adenosine-H_1-receptor interaction. Similarly, endogenous adenosine potentiated the ^{3}H-cyclic AMP response to HA in the cortical vesicular preparation (Table 6; McNeal et al., 1980; Newton and Hough, 1984). In the present study, inclusion of adenosine deaminase decreased the ability of H_1-antagonists to inhibit the ^{3}H-cyclic AMP response to HA (cf. Fig. 8 vs Fig. 14) suggesting that endogenous adenosine potentiated the response to H_1-receptor stimulation (see Discussion 2.3. for further details). These observations all suggest that H_1-receptor stimulation can potentiate the cyclic AMP response to endogenous adenosine in both slice and vesicular preparations of brain.

Whether addition of adenosine influences H_2-mediated cyclic AMP accumulation in different brain preparations is unknown. Dismukes et al., (1976b) demonstrated that the

^{14}C-cyclic AMP response to TEA (100 uM) was only sensitive to inhibition by metiamide (10 uM) in the absence of adenosine (0.1 mM). Perhaps the H_2-component was maintained but masked by the larger H_1-contribution to the TEA response seen in the presence of this nucleoside. This hypothesis might be supported by the observation that the ^{14}C-cyclic AMP response to 4-methyl-HA (100 uM) was sensitive to inhibition by metiamide (10 uM) in both the presence and absence of adenosine (Dismukes et al., 1976b). In the present study, endogenous adenosine did not appear to decrease the H_2-receptor mediated response to HA (Fig. 23) or dimaprit (Fig. 22). It is unknown however, how addition of higher adenosine concentrations might influence H_2- or H_1-mediated cyclic AMP accumulation in this vesicular preparation.

It seems likely that H_1-mediated cyclic AMP accumulation is calcium-dependent. In the current study, a combination of EGTA and adenosine deaminase eliminated the H_1-component in the HA response. Similarly, Daly et al., 1979, have shown that the ^{14}C-cyclic AMP response to HA (100 uM) in slices of guinea pig cerebral cortex was not inhibited by the H_1-antagonist brompheniramine (100 uM) in the presence of EGTA (2 mM) and the adenosine receptor antagonist theophylline (200 uM). However, in the presence of EGTA (2 mM), but in the absence of theophylline, approximately 50% of HA-stimulated ^{3}H-cyclic

AMP accumulation was blocked by brompheniramine (100 uM). Although this concentration of brompheniramine may have actions in addition to H_1-receptor antagonism, this latter result might imply that in the presence of adenosine H_1-receptor stimulated cyclic AMP accumulation can occur in a calcium-independent fashion. Another study in slices of guinea pig cortex demonstrated that the cyclic AMP response to a combination of dimaprit (100 uM) and TEA (100 uM), but not the response to dimaprit alone, was reduced by inhibition in calcium-free medium (Schwartz et al., 1980a). Since many other H_1-receptor mediated responses are probably calcium-dependent (see Introduction 5), it is attractive to accept the hypothesis that H_1-mediated cyclic AMP accumulation also requires this cation.

Applying considerations of the metactoid model to HA-mediated cyclic AMP accumulation in brain slice studies may unravel several anomalous observations. For example, in guinea pig cortical slices, although metiamide (100 uM) inhibited the ^{14}C-cyclic AMP response to HA by 50%, Rogers and Daly (1975) suggested that only H_1-receptors were coupled to ^{3}H-cyclic AMP accumulation. This conclusion appears to be based on two erroneous assumptions. Firstly, it was assumed to be impossible for the H_1-antagonist brompheniramine (1-100 uM) to inhibit 75% and for metiamide (100 uM) to inhibit 50% of the ^{14}C-cyclic AMP

response to HA (100 uM), since this would mean that greater than 100% of this HA response could be blocked by these agents. However, the present results show that when a fraction of the HA response is dependent on concomitent stimulation of both H_1- and H_2-receptors stimulation (see Fig.23) this is precisely the case. Secondly, a nonspecific action of metiamide was inferred from the observation that metiamide (1 uM) did not inhibit the cyclic AMP response to HA, yet brompheniramine (1 uM) inhibited the HA response by 75%. Why this latter finding constitutes lack of evidence for H_2-receptor participation in the HA response is obscure. Given that the pA_2 of metiamide at H_2-receptors is 6.0 and the EC_{50} of H_2-mediated cyclic AMP accumulation is 10 uM, it can be calculated that 1 uM metiamide would not significantly antagonize the response to 100 uM HA. This work was subsequently quoted (Daum et al., 1982) as evidence for the lack of H_2-participation in HA-mediated cyclic AMP accumulation in slices of guinea pig cortex. A more likely explanation for the findings of Rogers and Daly (1975), as suggested in the present study, would be to propose that a fraction of the H_1-response required activation of H_2-receptors for expression.

In a detailed pharmacological characterization of H_1-mediated cyclic AMP accumulation in guinea pig hippocampal slices (Palacios et al., 1978a), it seems

likely that the method used to calculate H_1-antagonist dissociation constants may have resulted in an overestimation of the true dissociation constants of these agents. Despite evidence that the H_1-component of the cyclic AMP response to HA was dependent on H_2-receptor activation, (e.g. metiamide caused parallel concentration-dependent shifts to the right in the entire HA concentration-response, while mepyramine caused a concentration-dependent shift to the right of only 50% of the cyclic AMP response to HA), Palacios et al. (1978a) obtained H_1-antagonist dissociation constants by fitting their data to an independent two site model. Failure to account for the interaction of H_1- and H_2-receptors in this analysis may have resulted in an overestimation of antagonist dissociation constants. This observation is not trivial, since these deviations in reported H_1-antagonist dissociation constants have been used to question the participation of H_1-receptors in this HA response (Johnson, 1982).

While a metactoid interaction between H_1- and H_2-receptor stimulation and cyclic AMP accumulation might be a common feature of HA-mediated cyclic AMP accumulation in intact preparations of brain, additional properties of H_1-antagonists may obscure the ability of these agents to inhibit H_1-receptor stimulated cyclic AMP accumulation. For example, the reported dissociation constant for

promethazine in inhibiting H_1-mediated potentiation of H_2-stimulated cyclic AMP accumulation in guinea pig hippocampal slice preparations (20-25 nM; Palacios et al., 1978a; Trung Tuong et al., 1980) was about an order of magnitude higher than the ability of promethazine to inhibit the H_1-mediated potentiation of H_2-mediated ^3H-cyclic AMP accumulation seen in the present study (1.05 nM, Table 7), or in inhibiting H_1-potentiation of the cyclic AMP response to exogenous adenosine (0.1 mM) in slices of guinea pig cortex (1.7 nM, Hill et al., 1981). It seems possible that this difference might be attributable to other action(s) of promethazine which vary between different brain regions. For example, promethazine is known to inhibit calcium-dependent phosphodiesterase (Levin and Weiss, 1976). It is possible that the relationship between cyclic AMP synthesis and metabolism varies among brain regions, such that inhibition of calcium-dependent phosphodiesterase by promethazine increases HA-mediated cyclic AMP accumulation in hippocampal slices, thus masking the ability of this antagonist to inhibit H_1-receptor mediated cyclic AMP accumulation in this brain region. In cortical vesicular or slice preparations, promethazine inhibition of cyclic AMP metabolism might be small relative to H_1-mediated cyclic AMP accumulation, and thus the inhibitory effect of this antagonist on H_1-mediated cyclic AMP accumulation would be apparent. These differences in the reported

dissociation constants for promethazine illustrate the need to determine the pharmacological characteristics of H_1- (and other) receptor-mediated responses through as many different techniques as possible, in order to preclude the possible misclassification of a receptor coupled to a given response.

4. Mechanisms and Implications of Histamine Receptors Coupled to Cyclic AMP Accumulation in Brain.

The similarities between HA-responsive cyclic AMP accumulation in brain slice and vesicular preparations implies that minimal destruction of CNS structural integrity, as found in the vesicular preparation under study, does not destroy the capacity for H_1-receptors to indirectly increase CNS cyclic AMP accumulation. Other than preliminary observations suggesting that the H_1-component of the ^{3}H-cyclic AMP response to HA is potentiated by adenosine and is calcium-dependent, very little is known about the mechanisms of either H_1- or H_2-mediated increases in ^{3}H-cyclic AMP. Based on available evidence from the literature, several mechanisms should be considered for both H_1- and H_2-stimulated ^{3}H-cyclic AMP accumulation. Several of these candidates are considered below.

H_2-receptor stimulation presumably increases ^{3}H-cyclic AMP through direct activation of adenylate cyclase in all brain preparations. In broken cell membrane preparations of brain, H_2-receptor stimulaton of adenylate cyclase is potentiated by free magnesium and GTP but inhibited by calcium (Kanof et al., 1977), and presumably acts through a receptor-linked guanyl nucleotide dependent mechanism (see Birnbaumer and Iyengar, 1982).

It is not clear whether H_2-receptors directly stimulate the conversion of ^{3}H-adenine-prelabeled ATP to ^{3}H-cyclic AMP in vesicular and brain slice preparations, i.e. whether this is the same response as that measured in broken cell membrane preparations using exogenous ATP as substrate. Specific activity changes in isotopically labeled cyclic AMP formed in response to HA (100 uM) and epinephrine (10 and 100 uM) have been reported to occur in guinea pig cortical slices (Chasin et al., 1973), suggesting that unidentified HA-receptor(s) also cause conversion of unlabeled ATP to cyclic AMP in this tissue. If this unlabeled ATP substrate pool is equivalent to exogenous ATP, as used in broken cell preparations, it is possible that an H_2-stimulated increase in unlabeled cyclic AMP could indirectly cause an increase in ^{3}H-cyclic AMP levels by competing with ^{3}H-cyclic AMP for metabolism.

Other H_2-mediated mechanisms, in addition to presumed adenylate cyclase activation, may also increase ^{3}H-cyclic AMP levels in the vesicular preparation. For example, an H_2-mediated increase in potassium conductance (see Green and Hough, 1984) might increase ^{3}H-cyclic AMP levels by indirectly inhibiting phosphodiesterase activity, as the preparation studied presently contains no phospho diesterase inhibitor. High extracellular potassium is known to increase ^{3}H-cyclic AMP levels in brain and also inhibited calmodulin-sensitive phosphodiesterase in brain

slices (Davis and Daly, 1978).

The possibility that H_2-receptor stimulation releases adenosine to the extracellular medium and that adenosine acts to permit expression of H_1-receptor stimulation, as suggested by others (Palacios et al. 1978a) seem unlikely. In guinea pig cortical brain slices, addition of IBMX (100 uM) (a phosphodiesterase and adenosine receptor antagonist), did not abolish the H_1-mediated ^{3}H-cyclic AMP response (Leigh et al., 1984), a predicted effect if H_1-mediated ^{3}H-cyclic AMP accumulation required concomitant stimulation of adenosine receptors. In the present study, in the presence of adenosine deaminase, it seems unlikely that sufficient adenosine could still be released to the medium to cause a measurable increase in ^{3}H-cyclic AMP levels through the potentiation of the response to H_1-stimulation. In a vesicular preparation of guinea pig cortex prepared and incubated in hepes buffer, I have obtained preliminary evidence that inclusion of 8-phenyl-theophylline (2 uM) (an agent reputed to be a specific adenosine receptor antagonist), with or without adenosine deaminase (2.5 U/ml) does not eliminate the ability of mepyramine (0.1 uM) to inhibit the ^{3}H-cyclic AMP response to HA (Newton and Hough, 1984). In contrast, Daly et al (1980) have shown that in the presence of 8-phenyl-theophylline (20 uM), the ^{3}H-cyclic AMP response to HA (100 uM) is not antagonised by the H_1-antagonist

d-brompheniramine (5 uM). This latter result might suggest that adenosine-receptor antagonism blocked H_1-mediated ^{3}H-cyclic AMP accumulation in this preparation. However, in the same study, in the presence of theophylline (Ø.2 mM), an adenosine receptor antagonist and potential phosphodiesterase inhibitor, the ^{3}H-cyclic AMP response to 1ØØ uM HA was inhibited by 67% by d-brompheniramine (1ØØ uM). Clearly, the inter-relationships among adenosine, adenosine-receptor activation, and HA-stimulated cyclic AMP accumulation in brain is still far from understood.

It is also unclear how adenosine increases ^{3}H-cyclic AMP levels in vesicular and brain slice preparations of brain. An extracellular adenosine receptor is believed to mediate these changes since studies in brain slices have indicated that inhibition of adenosine uptake potentiates the response to this nucleoside and this response is antagonised by adenosine receptor antagonists (see Introduction 2). However, adenosine does not activate adenylate cyclase in broken cell membrane preparations of rat cortex (Premont et al., 1979). Indeed this preparation contains an inhibitory adenosine receptor coupled to adenylate cyclase (Cooper et al., 198Ø), which does not appear to have been demonstrated in brain slice studies. Adenosine can however, stimulate adenylate cyclase in broken cell membrane preparations of rat

striatum (Premont et al., 1979). It seems that, as with H_1- and H_2-mediated cyclic AMP accumulation in brain, the effects of adenosine may also be divided into subclasses whose demonstration depends on the brain preparation and region under study.

Several mechanisms for H_1-receptor stimulated ^3H-cyclic AMP accumulation are also possible. The results from the present study do not exclude a direct H_1-receptor (or adenosine-receptor) mediated stimulation of cyclic AMP accumulation, through a putative calcium-dependent adenylate cyclase (see Cheung and Storm, 1982 for recent review). It is possible that H_1-receptor stimulation is directly coupled to ^3H-cyclic AMP synthesis in the vesicular preparation yet, in the absence of alternative stimuli leading to a relative increase in the synthetic rate, this increase in ^3H-cyclic AMP will not be seen. For example, an H_2-mediated increase in unlabeled cyclic AMP, through inhibiting phosphodiesterase, could reveal H_1-mediated stimulation of ^3H-cyclic AMP accumulation.

Other mechanisms may also be involved in H_1-mediated ^3H-cyclic AMP stimulation. For example, an H_1-mediated rise in cyclic GMP could, through competing with cyclic AMP for metabolism, indirectly increase cyclic AMP levels. Such a hypothesis is consistent with the observation that EGTA eliminates the cyclic GMP response to HA and reduces cyclic AMP accumulation in guinea pig cerebral cortical

slices (Schwabe et al., 1978). Alternatively, it seems possible that H_1-stimulated glycogenolysis could, by increasing limited substrate ATP pools, increase the cyclic AMP response to stimulation of receptors coupled to adenylate cyclase activation.

An H_1-mediated change in phosphatidylinositol turnover might also be linked to H_1-mediated changes in cyclic AMP accumulation. It has been suggested that H_1-receptor mediated stimulation of phosphatidylinositol turnover is involved in all H_1-mediated responses (Jones et al., 1979). The cyclic GMP response to HA may require H_1-receptor stimulation of phosphatidylinositol turnover, and both of these processes require calcium (Snider et al., 1984). Since H_1-stimulated cyclic AMP accumulation is also probably calcium-dependent, it is possible that H_1-mediated increases in cyclic AMP accumulation could also involve changes in phosphatidylinositol turnover. Possibly, H_1-receptor mediated changes in membrane phospholipid composition, resulting in an increase in membrane fluidity, might increase H_2-mediated cyclic AMP accumulation by increasing the frequency of coupling between H_2-receptors and adenylate cyclase.

It has been suggested that potentiation between putative neurotransmitters mediating cyclic AMP accumulation in brain involves different types of cells, i.e. different neuronal and/or glial populations, since

combinations of different neurotransmitters did not cause greater than additive cyclic AMP responses in cultured cell lines (see Dunwiddie and Hoffer, 1982). This hypothesis is not weakened by my study in the vesicular preparation, since H_1-receptor stimulation of one cell type, through causing the release of unknown agents, might cause potentiation of the response to H_2-receptor stimulation at a different site.

The metactoid nature of H_1-receptor stimulated ^3H-cyclic AMP accumulation should be considered in future studies designed to show the localization of HA-receptors coupled to cyclic AMP accumulation in brain. For example, the failure to detect significant HA-mediated cyclic AMP accumulation in cerebellar slices of guinea pigs (Schwabe et al., 1978), which contains high levels of H_1-receptor binding sites (Hill et al., 1978; Tran et al., 1978), might be because H_2-receptors are not coupled to cyclic AMP accumulation in this brain region. This does not exclude the possibility that H_1-receptor stimulation could potentiate the cyclic AMP response to other alternative direct stimuli, such as adenosine- or β-receptor stimulation. Similarly, changes in HA-responsive cyclic AMP levels following lesioning studies should be assessed for which HA-receptor, if any, was altered by this treatment. In addition, possible lesion-induced changes in endogenous adenosine and/or adenosine receptors should

also be assessed, since H_1-, and possibly H_2-receptor, stimulated changes in cyclic AMP accumulation might be influenced by changes in adenosine-dependent cyclic AMP generating systems. Failure to consider potential metactoid interactions might result in erroneous conclusions on the localization of HA-responsive cyclic AMP generating systems in brain.

5. Implications for Roles of Hormone Receptors Coupled to Cyclic AMP Accumulation in Brain and Peripheral Tissues.

Receptors for other neurotransmitters coupled to cyclic AMP accumulation resemble HA-receptors in several respects. For example, β-adrenergic receptors can directly stimulate adenylate cyclase activation in broken cell membrane preparations of brain, while α-receptor mediated increases in cyclic AMP have only been demonstrated in brain slice or vesicular preparations of brain. Thus, β- or α-adrenergic receptors coupled to cyclic AMP accumulation in CNS tissues show similar properties to H_2- and H_1-receptors, respectively.

In analogy with the interaction of HA-receptors seen in the present study, α-adrenergic receptor stimulation appears to potentiate the cyclic AMP response to β-receptor stimulation in some brain regions. In slices of rat cortex (see Daly et al., 1980b and 1981), hypothalamus (Daly et al., 1981; Palmer et al., 1973) and spinal cord (Jones, and McKenna, 1982a and b), and in a vesicular preparation of limbic forebrain (Blumberg et al., 1976; Horn and Phillipson, 1976), both α- and β-receptor antagonists inhibit the cyclic AMP response to noradrenaline, a mixed α- and β-receptor agonist. The cyclic AMP response to norepinephrine was blocked by about 70 - 80% by the β-antagonist propranolol and about 50% by

the α-antagonist phentolamine in rat cortical slices (Daly et al., 1980b; Schwabe and Daly, 1977a). In analogy with the current study, these observations suggest that 20 - 30% of the cyclic AMP response to α-receptor stimulation in rat cortical slices may depend on concomitant β-receptor stimulation. A larger dependency of α-receptor mediated cyclic AMP accumulation on β-receptor stimulation may pertain in rat hypothalamic slices, where either α- or β-antagonists blocked the cyclic AMP response to norepinephrine (50 uM) by about 80-90% (Palmer et al., 1973). In contrast, α-mediated cyclic AMP accumulation in rat spinal cord does not appear to depend on -receptor stimulation, where 70% of the cyclic AMP response to noradrenaline was inhibited by phentolamine and 30% by propranolol (Jones and McKenna, 1980a). Application of the metactoid sensitization model developed in the current study to catecholamine-stimulated cyclic AMP accumulation should aid in determining to what extent, if any, α-receptor mediated cyclic AMP accumulation is dependent on β-receptor stimulation in different brain regions.

Also like the H_1-response seen in the current study (Figs. 8 and 13), α-receptor stimulation potentiated the cyclic AMP response to endogenous adenosine in the guinea pig vesicular preparation (Daly et al., 1980). Similarly both α-receptor (Sattin et al., 1975) and H_1-receptor stimulation (Daum et al., 1982; Hill and Young, 1981a)

potentiated the cyclic AMP response to exogenous adenosine in guinea pig cortical slices. In rat spinal cord, α1-receptor stimulation potentiated the cyclic AMP response to adenosine (100 uM) (Jones, 1981), although α1-mediated cyclic AMP accumulation seen in the absence of exogenous adenosine was not reduced by adenosine deaminase (Jones and McKenna, 1980b). In the presence of adenosine deaminase (10 ug/ml), noradrenaline (100 uM) potentiated the cyclic AMP response to the adenosine receptor agonist 2-chloroadenosine (100 uM) in slices of rat cerebral cortex and hippocampus, but only additive effects were observed in other brain regions (Daly et al., 1981). These regional differences in the interaction of α-receptor stimulation and adenosine on cyclic AMP accumulation suggest that the ability of H_1-receptors to potentiate the cyclic AMP response to adenosine may not occur in all brain regions.

The ability of endogenous adenosine to increase the cyclic AMP response to α- or H_1-receptor stimulation also appears to vary among brain regions. Thus, addition of adenosine deaminase (10 ug/ml) did not reduce the cyclic AMP response to noradrenaline in slices of rat hippocampus (Daly et al., 1981) or spinal cord (Jones and McKenna, 1980b), although inclusion of this enzyme decreased this response in cortical slices (Daly et al., 1981). These observations are reminiscent of the cyclic AMP response to

HA seen in slices of guinea pig hippocampus or cortex. Endogenous adenosine is unlikely to contribute to H_1-mediated cyclic AMP accumulation in guinea pig hippocampal slices, since the cyclic AMP response to HA is completely blocked by cimetidine (Palacios et al., 1978a). In guinea pig cortical slices, removal of endogenous adenosine decreases the response to HA, probably by removal of adenosine-dependent H_1-mediated cyclic AMP accumulation (Hill et al., 1981a). These observations suggest endogenous adenosine concentrations and/or the ability of neurohormones to potentiate the cyclic AMP response to endogenous adenosine varies among brain regions.

The ability to demonstrate hormone-receptors indirectly coupled to cyclic AMP accumulation may depend on the elucidation of currently unidentified hormone-receptors directly coupled to cyclic AMP accumulation, at least in some brain regions. For example, vasoactive intestinal polypepide (VIP) stimulates cyclic AMP accumulation in brain slices, and this response may be potentiated by α- and/or H_1-receptor stimulation, in some brain regions and/or species. VIP increased cyclic AMP in slices from several regions of rat brain (Quik et al., 1978). In this study, the cyclic AMP responses to VIP (Ø.5 uM) plus either noradrenaline (1Ø uM), adenosine (5Ø uM) or prostaglandin E_1 (5 uM) were additive in slices of cortex, hypothalamus or striatum. Since α-receptor stimulation

potentiates the cyclic AMP response to endogenous adenosine and β-receptor stimulation in rat cortical slices (see above), it is unclear whether the response to noradrenaline seen in this study occurred through a direct stimulation of β-receptors or through α-receptor potentiation of the response to VIP and/or endogenous adenosine. Stimulation of α-receptors potentiated the cyclic AMP response to VIP in mouse cerebral cortical slices (Magistretti and Schorderet, 1985). Activation of H_1-receptors may also potentiate this response to VIP since mepyramine (Ø.1 - 1 uM) caused a concentration-dependent inhibition of the cyclic AMP response to a combination of HA (1ØØ uM) plus VIP (1 uM), while cimetidine (1ØØ uM) had no effect (Magistretti and Schorderet, 1985). This study supports the hypothesis (Daly et al., 1980a) that stimulation of α- and H_1-receptors can indirectly increase the cyclic AMP response to different hormone-receptors coupled to cyclic AMP accumulation.

The indirect effects of α- and H_1-receptor stimulation on cyclic AMP accumulation in brain may not be additive with each other. HA (1ØØ uM) did not potentiate the response to VIP (1 uM) plus norepinephrine (1ØØ uM) in mouse cortical slices, although both H_1- and α-receptors may potentiate the response to VIP (Magistretti and Schorderet, 1985). Similar observations have been

reported for HA and noradrenaline potentiation of the cyclic AMP response to adenosine in rat cerebral cortex (Schultz and Daly, 1973c). In this study, the H_1-antagonist diphenhydramine (Ø.Ø5 mM) had little effect on the cyclic AMP response to HA (1ØØ uM) plus noradrenaline (1ØØ uM) or adrenaline (1ØØ uM), and this response was abolished by α-antagonists (1ØØ uM). This lack of additivity of α- and H_1-receptor stimulation may be related to the mechanism(s) mediating the indirect effect of these receptors on cyclic AMP accumulation. Pharmacological characterization of combination studies with H_1- and α-agonists, in the presence of a direct stimulus such as adenosine, may aid in elucidating the stimulus-response relationships governing the cyclic AMP response to these indirect stimuli.

The calcium-requirement for neurohormone-stimulated cyclic AMP accumulation varies among brain regions and species. Adenosine-mediated cyclic AMP accumulation was abolished by incubation in calcium-free medium or acute (2 min) incubation with EGTA (2 mM), in slices of rat spinal cord (Jones, 1981). In contrast, acute (2 min) incubation with EGTA (2 mM) either increased or did not alter the response to adenosine-receptor agonists in guinea pig or rat cortical slices (Schwabe and Daly, 1977; Schwabe et al., 1978). Prolonged (3Ø min) incubation in calcium-free buffer potentiated the cyclic AMP response to

noradrenaline (Ø.1 mM) in slices of guinea pig (Ohga and Daly, 1977) or rat (Schwabe et al., 1978) cortical slices, an effect which may have been due to increased release of endogenous adenosine (Schwabe et al., 1978). Acute (2 min) incubation with EGTA (2 mM) abolished the response to α- but not β-receptor agonists in rat cortical slices (Schwabe and Daly, 1977). In contrast, the cyclic AMP response to noradrenaline in guinea pig cerebellum, which appeared to be exclusively mediated by β-receptor stimulation, was calcium-dependent (Ohga and Daly, 1977). Thus, calcium appears to regulate the ability of H_1- (see Discussion 2.3) and α-receptors to potentiate the cyclic AMP response to directly acting stimuli, in the brain regions studied. The cyclic AMP response to presumed directly acting agonists may also be modulated by calcium in some brain areas. In this regard, although H_2-receptor mediated ^{3}H-cyclic AMP accumulation in the guinea pig cortical vesicular preparation appears to be unaltered by calcium removal (Fig. 22 and 23), H_2-mediated cyclic AMP accumulation in other brain regions may be modulated by calcium.

Prostaglandins of the E series may be required for α-receptor stimulated cyclic AMP accumulation in brain. Inclusion of prostaglandin synthetase inhibitors eliminated the ability of α-adrenergic agonists to potentiate the ^{14}C-cyclic AMP response to adenosine, but

had no effect on the response to β-receptor stimulation, assessed either in the absence of calcium or in the presence of α-receptor antagonism, in slices of rat cerebral cortex or hypothalamus (Partington et al., 1980). Inclusion of prostaglandin E_2 (0.1 uM), in the presence of indomethacin (100 uM), restored the ability of α-agonists to increase ^{14}C-cyclic AMP accumulation in rat cerebral cortical slices but had no effect on the ^{14}C-cyclic AMP response to β-agonists (Partington et al., 1980). Therefore, the indirect cyclic AMP response seen in response to α-receptor stimulation does not appear to occur through receptor-mediated release of prostaglandins, which could then directly stimulate cyclic AMP accumulation (Dismukes and Daly, 1975). A possible relationship between prostaglandins and H_1-mediated cyclic AMP accumulation merits investigation.

Interactions between different hormonal receptors initiating cyclic AMP accumulation are not limited to brain. Muscarinic receptor stimulation potentiates the cyclic AMP response to VIP in the cat submandibular gland (Enyedi et al., 1982; Enyedi and Fredholm, 1984). Like studies on potentiative interactions in CNS tissues, the potentiative effects of muscarinic stimulation on VIP-induced cyclic AMP accumulation in the cat submandibular gland were abolished by inclusion of EGTA in the incubation medium (Enyedi and Fredholm, 1984). Muscarinic

receptor stimulation also potentiated the cyclic AMP response to dopamine in cat retina (Brown and Reitow, 1981). However, this effect was not eliminated by inclusion of EGTA (Brown and Reitow, 1981). It seems possible that both H_1-, α-adrenergic and muscarinic-receptor stimulation act through metactoid sensitization to increase cyclic AMP accumulation in responsive tissues.

Combinations of different transmitters also cause greater than additive responses in peripheral tissues. For example, nucleotide contraction of the isolated guinea pig vas deferens is potentiated by noradrenaline, through α-adrenergic receptor stimulation (Holck and Marks, 1978; Kazic and Milosavljevic, 1980). Adenosine and AMP had no effect on contraction in the absence of noradrenaline, while in its presence adenosine produced a significant contaction and responses to ATP and ADP were potentiated (Holck and Marks, 1978). A similar potentiative interaction may also occur in guinea pig seminal vesicles, where ATP-induced contractions were enhanced by exogenously applied noradrenaline, or by hypogastric nerve stimulation (Nakaniski and Takeda, 1973). Thus the metactoid sensitization model developed in the current study may have applications in the elucidation of hormone-receptor interactions coupled to both biological and biochemical responses in peripheral tissues.

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