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Inhibition and stimulation of adenylyl cyclase by 5-HT receptors in guinea pig and rat hippocampal membranes: Classification of the receptors and GTP-binding proteins mediating the responses

De Vivo, Michael, Ph.D.

City University of New York, 1987

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INHIBITION AND STIMULATION OF ADENYLYL CYCLASE BY 5-HT RECEPTORS IN GUINEA PIG AND RAT HIPPOCAMPAL MEMBRANES: CLASSIFICATION OF THE RECEPTORS AND GTP-BINDING PROTEINS MEDIATING THE RESPONSES.

by Michael De Vivo.

A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
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1987

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ABSTRACT

INHIBITION AND STIMULATION BY 5-HT RECEPTORS
OF ADENYLYL CYCLASE IN GUINEA PIG AND RAT HIPPOCAMPAL MEMBRANES:
CLASSIFICATION OF THE RECEPTORS AND GTP-BINDING
PROTEINS MEDIATING THE RESPONSES.

by Michael De Vivo.

Adviser: Saul Maayani, Ph. D.

In guinea pig hippocampal membranes, 5-HT has a net stimulatory or inhibitory effect on adenylyl cyclase (AC) activity depending upon the degree of activation of the cyclase by forskolin. In the absence of forskolin, or in the presence of low concentrations of forskolin, 5-HT stimulates AC activity, whereas in the presence of high (micromolar) concentrations of forskolin, 5-HT inhibits AC activity.

Inhibition of AC by 5-HT is saturable and follows simple Michaelis-Menten kinetics. Antagonists competitively shift the concentration-response curve to 5-HT to the right with no reduction in the maximal percentage of inhibition. These data suggest that inhibition is mediated by a single population of receptors. Pharmacological classification of the inhibition demonstrates that the effect is mediated by a receptor homologous with the 5-HT_{1A} binding site. 5-HT_{1A} receptor-mediated inhibition of AC is also measurable in membranes from rat cerebral cortex

and hippocampus.

Stimulation of AC activity by 5-HT is mediated by two distinct populations of receptors, one population that is classified as 5-HT_{1A}, another that is designated as R_L (Shenker et al., 1985). Reducing the membrane concentration in the assay results in a reduction of the contribution of the 5-HT_{1A} receptor to the stimulation, permitting pharmacological analysis of the R_L-mediated response. Based on the affinities of the antagonists spiperone, fluphenazine and mianserin, the stimulation is mediated by a receptor homologous with the receptor that stimulates AC in infant rat collicular membranes (Nelson et al., 1980a,b).

Inhibition of adenylyl cyclase activity by 5-HT_{1A} receptor-stimulation requires micromolar concentrations of GTP and Mg^{++}. In vivo administration of pertussis toxin abolishes the 5-HT-mediated inhibtion of adenylyl cyclase activity in membranes. These data indicate that the inhibitory effect is mediated by a GTP-binding protein, presumably G_i. Stimulation of adenylyl cyclase activity requires GTP and millimolar concentrations of Mg^{++}. 5-HT receptor-stimulation results in a reduction in the EC_{50} value for Mg^{++} in stimulating adenylyl cyclase, a characteristic of G_s proteins (Iyengar and Birnbaumer, 1982).

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I. LITERATURE REVIEW

I.1 Introduction

Membranes effectively insulate cells from their environment, yet cells must be able to respond to changes in their surroundings and coordinate their actions with other cells. Therefore, cells have developed systems to detect and respond to extracellular information. Agents that influence cell physiology are drugs, and one method by which drugs influence cell physiology is to bind to signalling devices (receptors) in cell membranes. Receptors transmit information to the interior of the cell by various transduction mechanisms, including changing conductances of ions across membranes or stimulating formation of second messengers inside cells.

The subject of this dissertation is the study of receptors for 5-hydroxytryptamine (5-HT; serotonin) that inhibit adenylyl cyclase (AC) activity in guinea pig and rat hippocampal membranes. The pharmacology, regulation, and characteristic effects of these receptors with respect to GTP and Mg^{++} are examined. The results from these studies will be discussed with respect to 5-HT receptor classification, receptor transduction mechanisms and possible physiological consequences of 5-HT receptor-mediated changes in adenylyl cyclase activity. This section reviews 5-HT pharmacology and receptor transduction mechanisms with particular emphasis on 5-HT receptors coupled to adenylyl cyclase.

1.2 Classification of 5-HT receptors

During the middle of the 19th century, physiologists injected defibrinated blood into dogs and measured changes in the dogs' vascular resistance (Ludwig and Schmidt, 1868 as referenced in Page, 1954). Defibrinated blood contains physiologically active quantities of 5-HT, therefore these measurements were the first ever made of a 5-HT receptor-mediated response and represent the beginning of 5-HT pharmacology. Page (1954) summarized the effect of 5-HT on vascular responses in dogs as follows: "The vascular response to 5-HT is highly variable ... the response seems well described by the term, '*Amphibaric*' ,... since it is usually both pressor and depressor, but may be only one or the other". In many tissues, 5-HT elicits variable and even opposing responses. For example, 5-HT can both stimulate and inhibit adenylyl cyclase activity in hippocampal membranes, and can increase and decrease the probability of pyramidal cell discharge in the hippocampus. The variable effects of 5-HT may indicate that multiple types of 5-HT receptors are present in these tissues, or that 5-HT receptors are coupled to multiple effectors, complicating characterization of 5-HT receptor-mediated responses.

The contractility of vascular systems was used as a bioassay to monitor the purification of 5-HT. Therefore, 5-HT receptors were used to monitor the purification of the ligand 5-HT much like high affinity 5-HT receptor ligands are now used to monitor the purification of 5-HT receptors (Ransom et al., 1986b). Rapport, Green and Page (1948) isolated 5-HT from blood serum and Rapport (1949) determined its structure. Erspamer and Asero (1952) independently determined the structure of enteramine, a hormone isolated from the g.i. tracts of mammals, and found it to be identical to

5-HT. Using various intact organ preparations, the distribution of 5-HT among tissues and species was mapped. 5-HT is present in many invertebrates (Welsh, 1957) where it is found in such diverse roles as a neurotransmitter in clam hearts and as a component of wasp venom. In rats, the distribution is widespread in peripheral tissues with highest concentrations in the g.i. tract (enterochromaffin cells), blood (platelets), kidney and spleen (Page, 1954).

The discovery of 5-HT in brain tissue (Twarog and Page, 1953), and the the fact that the hallucinogen lysergic acid diethylamide (LSD) antagonizes 5-HT-induced contractions of rats' uterus, lead to the proposal by Wooley and Shaw (1954) and Gaddum (1953) that 5-HT might be involved in mental processes. In addition to LSD, drugs that affect the release (i.e. reserpine) or interfere with the synthesis or metabolism of 5-HT (Udenfriend et al., 1957) produce behavioral effects in animals (Shore et al., 1957), further supporting a role for 5-HT as a neuroactive substance. It was difficult to quantitate the effects of 5-HT in brain tissue at the time, hindering research on the effects of 5-HT in brain. Wooley and Shaw (1957) attempted to use changes in cell size in response to 5-HT as a pharmacological assay. Gaddum and Vogt (1956) investigated the psychoactive effects of LSD and 5-HT in cats by measuring changes in behavior after administration of 5-HT and LSD into ventricles. In a courageous attempt to quantitate the effects of LSD in man, they reported the following:

> "One of us took, on four occasions, 30-86 μg LSD by the mouth and experienced some of the known effects of this drug, such as a feeling of irresponsibility and euphoria, increased awareness of shapes and colours, shimmering of peripheral vision, and a dreamy feeling that sensations did not represent real objects. These

> subjective effects were accompanied by apparent intoxication and a loss of the power of concentration. Groups of 8 digits were read out from a table of random numbers and the subject tried to repeat them immediately. The normal average score in this test was nearly 100%, but under the action of LSD the score fell to a low figure and fatigue developed rapidly. There was no obvious incoordination but some loss of the power of reproducing a simple drawing. These effects reached a maximum in about 1 $^1/_2$ hr. and then gradually disappeared during the next 3-4 hr."

Gaddum and Hameed (1954) postulated the existence of two tryptamine receptors in peripheral tissues, a smooth muscle receptor found in the smooth muscle of rat's uterus and rabbit's ear artery that is readily antagonized by LSD, and a receptor located on ganglia in the guinea pig ileum that is insensitive to LSD and marked by a rapid desensitization to 5-HT. Later, Gaddum and Picarrelli (1957) demonstrated that both types of responses were present in guinea pig ileum. The smooth muscle receptor was designated "D" and the ganglionic receptor was designated "M" because the responses were blocked by dibenzyline and morphine, respectively. These drugs are not competitive antagonists of 5-HT receptors, and therefore are of limited usefulness in characterizing 5-HT receptors in other tissues.

Receptor classification in the CNS is more difficult than in peripheral preparations because of the difficulty in obtaining quantifiable responses in response to known concentrations of agonists. Roberts and Straughan (1967) demonstrated the existence of two types of 5-HT receptor in cerebral cortex: a receptor that increased neuronal firing and was blocked by LSD, methysergide and cinanserin, and a receptor that decreased firing

and was not blocked by these drugs. Aghajanian (1981) divided functional 5-HT receptors in brain into three groups: S1, S2 and S3. S1 receptors are present on facial and spinal (White and Neuman, 1980) motoneurons. This receptor has no effect on the firing rate of neurons itself (i.e. it is not excitatory or inhibitory) but facilitates the effect of excitatory amino acids, such as glutamate (McCall and Aghajanian, 1979). The S1 receptor is blocked by cyproheptadine, metergoline and methysergide. LSD and mescaline, but not nonhallucinogenic ergot-derivatives like lisuride and methysergide, sensitize these receptors to the actions of 5-HT and norepinephrine for many hours (Aghajanian, 1981). The S2 receptor is present on raphe neurons and mediates inhibition of raphe firing, possibly by opening a K^+ conductance (Aghajanian and Lakoski, 1984). LSD and lisuride are potent agonists at this receptor, as are recently developed 5-HT_{1A} agonists (see below). S3 receptors are present postsynaptically on forebrain neurons that receive dense serotonergic input. Activation of these receptors suppresses neuronal firing. LSD is a weak agonist at this site and no antagonists have yet been reported for this receptor.

Lack of selective 5-HT receptor antagonists hampered characterization of 5-HT receptor-mediated responses. No scheme emerged for 5-HT receptor classification other than the "D" and "M" classification. The development of selective, competitive antagonists for "D" receptors, (ketanserin; Leysen et al., 1982) and "M" receptors (MDL 72222 ; Fozard, 1984) were key steps in the emergence of a classification scheme for 5-HT receptors. Another important development was the technique of rapid filtration binding which allowed characterization of 5-HT binding sites in brain. 5-HT binding sites were divided into two categories: sites labeled by $[^3H]$5-HT (5-HT_1) and $[^3H]$spiperone (5-HT_2) (Peroutka and Snyder, 1979).

In order to provide a framework for 5-HT receptor classification, a committee of researchers in the field of 5-HT pharmacology delineated the criteria to be met in order to classify a response as mediated by one of three receptor types, designated 5-HT_1, 5-HT_2 (D) and 5-HT_3 (M) (Bradley et al., 1986). These criteria are: for 5-HT_1, the response should be elicited by 5-carboxamidotryptamine (5-CT), it should be blocked by methiothepin, methysergide should be a potent antagonist or agonist and the response should not be blocked by ketanserin or MDL 72222; for 5-HT_2, the response should be blocked by ketanserin and methysergide and not blocked by MDL 72222; for 5-HT_3, the response should be blocked MDL 72222 and ICS 205-930 and not blocked by ketanserin. Typical responses to 5-HT_2 activation include: contraction of smooth muscle (Apperley et al., 1976), platelet aggregation (De Clerck et al., 1984), 5-HTP-induced head twitches in rats (Malick et al., 1977) and rat paw edema (Ortmann et al., 1982). Activation of 5-HT_2 receptors induces phosphatidylinositol turnover in human platelets (de Chaffoy de Courcelles et al., 1985) and in rat brain cortical slices (Conn and Sanders-Bush, 1984). Typical responses to 5-HT_3 receptor activation include: enteric neuronal transmitter release (Richardson et al., 1985), depolarization of ganglionic neurons (Fozard et al., 1985), and pain in blister base preparations (Richardson et al., 1985).

5-HT_1 binding sites are subdivided into 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} sites (Hoyer et al., 1985). The affinities of agonists in inhibiting release of 5-HT from rat brain cortical slices are significantly correlated with their affinity for 5-HT_{1B} sites (but also for 5-HT_{1A} sites) (Engel et al., 1986). The 5-HT_{1C} sites may correspond to receptors mediating phosphatidyl-inositol turnover in rat choroid plexus (Conn et al., 1986). The 5-HT_{1A} binding site is characterized by a high affinity for spiperone (Pedigo et

al.,1981), 8-hydroxy-2-(di-n-propylamino)tetralin (PAT) (Middlemiss and Fozard, 1983), buspirone (Glaser and Traber, 1983) and 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine (PAPP) (Ransom et al., 1986a,b). PAT has been especially useful in identifying responses that may be mediated by receptors homologous with the 5-HT_{1A} binding site.

Biochemically, PAT inhibits forskolin-stimulated adenylyl cyclase activity (De Vivo and Maayani, 1985,1986; see discussion) and vasoactive intestinal peptide-stimulated cAMP accumulation in cultured murine striatal and cortical cells (Weiss et al., 1986), and stimulates basal adenylyl cyclase activity in homogenates from guinea pig (Shenker et al., 1985) and rat (Markstein et al., 1986) hippocampi. Behaviorally, PAT induces forepaw treading and flat body posture in reserpinized rats, and these effects are blocked by spiperone, (-)pindolol and methiothepin, indicating that these responses are mediated by $5HT_{1A}$ receptors (Tricklebank et al., 1985). PAT and RU 24969 (a high affinity 5-HT_{1A} agonist) induce hypothermia in rats, and the effect is blocked by pindolol, sudggesting that this is also a 5-HT_{1A} receptor-mediated response (Tricklebank et al.,1985,1986). PAT and lisuride increase the acoustic startle response amplitude in rats, and the effect is blocked by methiothepin (Svensson, 1985).

Two peripheral preparations responsive to PAT have been identified: contraction of canine basilar artery (Taylor et al., 1986) and inhibition of transmitter release from guinea pig enteric neurons (Fozard and Kilbinger, 1985). The canine basilar artery preparation may not be useful for pharmacological classification because the response may be mediated by multiple receptors. The inhibition of transmitter release in guinea pig ileum was measured by incubating ileal tissue with labeled [^{3}H]choline,

electrically stimulating the tissue, and monitoring the release of the label from cholinergic neurons. PAT inhibits electrically evoked release, and the concentration-response curve to PAT was shifted to the right by low concentrations of by (-)pindolol, methiothepin, metergoline and buspirone, indicating that the response is mediated by 5-HT_{1A} receptors.

PAT produces electrophysiological effects in CNS tissue, such as a hyperpolarization of hippocampal pyramidal cells (Andrade and Nicoll,1985), a decrease in population spike amplitude in the CA1 region of the hippocampal slice (Beck et al., 1985) and an inhibition of raphe neuronal firing (De Montigny and Blier, 1983). 5-HT_{1A} receptors may be coupled to K^+ channels in hippocampal pyramidal cells independently of cAMP-mediated mechanisms (Andrade et al., 1986). Buspirone, LSD, lisuride and PAPP all inhibit raphe firing and are potent agonists at the 5-HT_{1A} receptor, suggesting that these receptors are homologous (Sprouse and Aghajanian, 1986; Aghajanian, 1981; VanderMaelen and Wilderman, 1984). A high density of PAT binding sites was measured at the dorsal raphe nucleus (Marcinkiewicz et al, 1984), and degeneration of of serotoninergic cell bodies by 5,7-DHT lesioning reduced the specific binding of PAT (Verge et al., 1985), supporting the contention that autoreceptors at the raphe cell body are of the 5-HT_{1A} type. However, a recent abstract (Steinberg et al., 1986) indicates that methiothepin does not antagonize the inhibition by 5-HT_{1A} agonists of raphe neuronal firing, , complicating classification of the receptor that mediates this response as 5-HT_{1A}.

An important caveat concerning 5-HT receptor classification should be introduced at this point. Contractions of rat jugular vein, rabbit aorta and rat caudal artery are all classified as 5-HT_2 responses, yet all show differences in antagonism by methysergide, trazodone, ketanserin and

spiperone (Leff and Martin, 1986). These authors suggest that classification should more appropriately be done with antagonists that are structurally similar to agonists in order to ensure that the antagonists are acting at the same site as the agonist. The development of such antagonists may also prove useful in explaining discrepancies in 5-HT_3 and 5-HT_1 receptor classification. For example, ICS 205-930 has markedy different affinites for 5-HT_3 receptors in different tissues (Richardson et al., 1985), and, as mentioned above, methiothepin does not antagonize the response to 5-HT_{1A} agonists in the raphe.

1.3 5-HT-mediated increases in cAMP accumulation

Cook, Lipkin and Marham (1957) investigated the breakdown products of ATP in the presence of barium hydroxide and identified a product that contained adenine, ribose and a phosphate group. At the same time, a factor was isolated from hepatic tissue that accumulated in response to epinephrine and glucagon (Sutherland and Rall, 1957). Both groups were put in touch with each other, exchanged samples, and determined that the substances they were studying were identical. The structure of cAMP was elucidated (Lipkin et al., 1959). Sutherland and Rall realized the potential importance of cAMP as an intracellular second messenger mediating the effects of receptor stimulation, not only in canine liver tissue but in many other tissues and species as well. Mansour et al. (1960), in one of the first reports of hormone-sensitive adenylyl cyclase activity in a tissue other than canine liver, reported that 5-HT stimulated cAMP accumulation in particulate preparations from the flatworm, *Fasciola hepatica* This system

is possibly the most responsive of any hormone-sensitive AC system yet discovered in terms of fold-stimulation (see below).

In reviewing the effects of 5-HT on cAMP, the following considerations should be noted. First, most of the experiments discussed in this section were reported before the characterization of 5-HT receptors negatively coupled to AC. Therefore, drugs decreasing 5-HT-stimulated AC activity were assumed to be 5-HT receptor antagonists, when in fact these drugs could act at receptors negatively coupled to AC. This is particularly true for LSD and methysergide, since both are potent in inhibiting AC in guinea pig membranes (see results) and were used routinely to characterize 5-HT receptors. Second, compensatory mechanisms exist in intact cells to rapidly lower cAMP levels after stimulation, such as activation of phosphodiesterases, cAMP efflux, and desensitization (Harden, 1983). These compensatory mechanisms tend to obscure the relationship between concentration of drug and response. Third, 5-HT-mediated increases in cAMP in intact cells or tissues may be secondary to other effects in the cell, such as Ca^{++} mobilization. Ca^{++} can stimulate or inhibit AC activity and activate phosphodiesterases (Rasmussen and Barret, 1984). Therefore, receptors that mobilize Ca^{++} can stimulate or inhibit cAMP formation without being directly coupled to AC, particularly when the increase is small relative to other stimulators of AC in the same tissue. This is less likely to be a problem with measurements of AC activity in membrane preparations, because the problem with interference of other effector systems is less likely to occur in a washed, broken cell preparation, especially in the presence of a chelator of Ca^{++} such as EGTA.

In the review that follows, when an IC_{50} value of an antagonist has been reported, I have converted the value into a K_i value by the method of Cheng

and Prusoff (1973), (i.e. I divided the IC_{50} by [1 + [A]/EC_{50}], where [A] represents the concentration of agonist and IC_{50} is the concentration of antagonist that reduces the maximal agonist stimulation by 50%). In order to give an indication of the reliability of the data reported in some experiments, I calculated a signal to noise ratio (SNR) as the ratio of the magnitude of the response and the standard error of the mean of the measurement of basal activity. For example, in hippocampal membranes, 5-HT inhibits from 150 to 110 pmols cAMP/mg protein/min., a difference of 40 pmols, with an average SEM of approximately 1.5 pmol/mg/min. The SNR is 40/1.5, or 27. In general, it is difficult to fit concentration-response curves to experimental data with an SNR of less than 10. Therefore, EC_{50} values obtained from systems in which the SNR is less than 10 should be used with caution.

As mentioned previously, 5-HT is widespread in invertebrates, and 5-HT-stimulated AC activity is also present in many invertebrate tissues. 5-HT-sensitive adenylyl cyclase of the liver fluke, *Fasciola hepatica*, has been especially well studied. The liver fluke has the highest SNR of any system, approximately 150, estimated from the data reported by Northup et al. (Northup and Mansour, 1978). Even a partial agonist with an intrinsic activity of 0.1 could be measured in such a system with no difficulty. 5-HT and LSD stimulate adenylyl cyclase activity and cAMP formation in fluke particles, and both drugs stimulate rhythmic motility in the intact animal (Abrahams et al., 1976). Stimulation of AC activity by 5-HT or LSD is increased synergistically by GTP, and the stimulation desensitizes upon prolonged exposure of the animal to 5-HT or by incubation of the membranes with 5-HT (Mansour, 1979; McNall and Mansour, 1984). The effects of 5-HT and LSD on adenylyl cyclase and motility are blocked by bromo-LSD (BOL)

(Northup et al., 1978). Stimulation of cAMP production is coincident with glycolysis, glycogenolysis and activation of phosphofructokinase and protein kinase activity (Mansour, 1979). 5-HT acts synergistically with forskolin to increase AC activity and increase motility (McNall and Mansour, 1983). Therefore, cAMP production in *Fasciola hepatica* (and possibly in the blødd fluke *S. mansoni*) may coordinate carbohydrate metabolism and motility. This 5-HT receptor does not resemble any of the three classes of receptor defined by Bradley et al., since methiothepin does not potently antagonize the response (IC_{50} = 1 µM; K_i = 0.7 µM) and BOL is a potent antagonist (K_i = 10 nM), which it is not at 5-HT_3 receptors (Nash et al., 1984). Ketanserin and spiperone are also ineffective in antagonizing the response. According to Mansour, liver flukes must be motile to survive, and selectively blocking the effect of 5-HT receptors that control motility or carbohydrate metabolism without affecting the host would be of enormous therapeutic value. It is estimated that 180 million people are infected with blood flukes, mostly in developing countries, but few research efforts have been made to develop drugs to combat these infections.

In heart muscle of the clam, *Mercenaria mercenaria*, 5-HT stimulates AC activity in membranes and cAMP accumulation in intact tissues (Higgins, 1977). When contractility of the myocardium desensitizes to the presence of 5-HT, the tissue also desenstitizes specifically to the effect of 5-HT on cAMP production, but not to a cardioactive peptide that also stimulates cAMP formation in this tissue, suggesting that the rise in cAMP mediates the inotropic effect of 5-HT. Similarly, 5-HT increases the contractility of heart muscle from the sea snail *Aplysia*, and, in the same tissue, increases cAMP accumulation, and both effects of 5-HT are enhanced by phosphodiesterase inhibitors and blocked by methysergide (Sawada et al.,

1984). In blowfly salivary glands, 5-HT stimulates cAMP accumulation and fluid secretion, and the effects of 5-HT on cAMP correlate with its effects on secretion (Berridge and Heslop, 1981). 5-HT facilitates neurotransmitter release at the crayfish neuromuscular junction, and this effect is enhanced by phosphodiesterase inhibitors (Enyeart, 1981). 5-HT-mediated relaxation of molluscan smooth muscle is mimicked by cAMP (Marchand-Dumont and Baguet, 1975). In R15 neurons of *Aplysia*, 5-HT-mediated increases in cAMP and hyperpolarization of the neurons are apparently homologous, and the effect of 5-HT is enhanced by phosphodiesterase inhibitors and mimicked by cAMP analogs (Drummond et al., 1980). Hyperpolarization of neurons may be mediated by an increased K^+ conductance. In cockroach thoracic ganglia homogenates, 5-HT stimulates AC activity with an EC_{50} of 0.5 µM and LSD inhibits this activity with an EC_{50} of approximately 10 nM (Nathanson and Greengard, 1973, 1974), similar to the EC_{50} value of LSD in inhibiting forskolin-stimulated activity in guinea pig hippocampus (see results). At higher concentrations, LSD is a partial agonist at this receptor. Other invertebrate preparations that respond to 5-HT with an increase in cAMP or AC activity include: ganglia and muscle of the leech (Biondi et al., 1982); annelid nervous sytem (Robertson and Osborne, 1979), *Aplysia* gill homogenates, (Weiss and Drummond, 1981); *Aplysia* and Helix neurons (Levitan, 1978), planaria (Franquinet et al., 1978), and abalone gill homogenates (Gentleman and Mansour, 1977). The effects of 5-HT and cAMP on *Aplysia* sensory neurons is reviewed in more detail in section I.4.

Classification of 5-HT receptors mediating cAMP accumulation in peripheral tissues and the physiological significance of those increases is, in most cases, speculative. In all cases, the EC_{50} of 5-HT is approximately 1 µM. 5-HT decreases glomerular filtration rate and increases cAMP content

4-5 fold in rat kidney cortical slices, and the effect is blocked by 100 μM methysergide or cinanserin (Shah et al., 1979) and selectively inhibited by α_2-adrenergic receptor stimulation (Umemura et al., 1986). In porcine vena cava, 5-HT, apparently through a 5-HT_1 receptor, increases cAMP content and mediates smooth muscle relaxation (Trevethick et al., 1984, 1986). 5-HT inhibits amino acid release and increases cAMP accumulation in rat epitrochlaris muscle (Garber, 1977), and both effects of 5-HT are reduced in rats with experimental chronic uremia (Garber, 1978). 5-HT promotes glycogenolysis and formation of phosphorylase a in this tissue, and all of the effects of 5-HT are blocked by micromolar concentrations of cyproheptadine, methysergide and propranolol (Ezrailson et al., 1983). Epinephrine produces similar effects in this muscle, but the effects are not blocked by methysergide. The effects of 5-HT are probably not indirect effects of released norepinephrine, since depletion of endogenous catecholamines in vivo did not effect the subsequent responsiveness of the system to 5-HT, but the indirect effects of tyramine were abolished (Ezrailson, 1983). In rat adrenal zona glomerulosa cells, 5-HT stimulates formation of cAMP and steroidogenesis (Albano et al., 1974), the two effects of 5-HT occur over the same concentration range of 5-HT and the effects of 5-HT are mimicked by agents that increase cAMP in the tissue (Fujita et al., 1979). The effects of 5-HT were completely blocked by 1 μM methysergide but only partially inhibited by 1 μM ketanserin (Williams et al., 1984), suggesting that the receptor may be of the 5-HT_1 class (or, alternatively, that ketanserin is a noncompetitive antagonist at this receptor). Other tissues in which 5-HT stimulates cAMP formation and possible physiological roles (when postulated by the authors) for the increase in cAMP are: rat brown fat, lipolysis (Fain et al., 1973; Yoshimura

et al.,1969); rabbit corneas, chloride transport (Neufeld et al., 1982, 1984); parathyroid adenoma tissue, release of parathyroid hormone (Zimmerman et al., 1980); rabbit retina (Blazynski et al., 1985); cultured calf aorta smooth muscle cells (Luchins and Makman, 1980); human medullary thyroid carcinoma (Matsukura et al., 1981); and rat mast cells (Gripenberg et al., 1974).

In rat and guinea pig heart slices, 5-HT elicits a small rise in cAMP accumulation, but the effect can be distinguished from its effect on cardiac contractility (Benfey et al., 1974; Higgins et al., 1981). The cAMP increase probably results from a release of norepinephrine by 5-HT_3 receptors (Fozard and Mobarok Ali, 1978). Therefore, while 5-HT may regulate heart contractility by a cAMP-dependent mechanism in invertebrates such as *Aplysia* and clams, in mammalian systems (and probably other vertebrates as well) ß-adrenergic receptors serve this purpose. Similarly, while 5-HT, but not epinephrine, has an important role in carbohydrate metabolism in invertebrates like liver flukes, the role of 5-HT in rat skeletal muscle carbohydrate metabolism is small relative to epinephrine.

In slices from various regions of mammalian brain, 5-HT was reported to have little or no effect on cAMP accumulation. Kakiuchi and Rall (1968, a,b) reported that 5-HT produced a small (with respect to norepinephrine and histamine) but significant increase in cAMP content in rabbit cerebellar slices, but not in slices of rabbit cerebral cortex. In slices from cerebral cortex, cerebellum, and hypothalamus from rabbit, rat, cat and monkey, Forn and Krishna (1971) reported no effect of 5-HT after a 10 minute incubation, despite the fact that these preparations produced robust responses to norepinepinephrine and/or histamine, up to 20-fold in some cases. In rat hypothalamic slices, Daszuta et al (1979), measured a decrease in cAMP

accumulation in the presence of 10^{-7} M 5-HT but, at higher concentrations of 5-HT, cAMP accumulation occurred, in contrast to the results from Forn and Krishna. Daszuta reported that the maximal increase to 5-HT occurred with tissue from 7 day old rats and with an incubation time of 6 minutes. Possibly the shorter time of incubation produced an effect that was not observed after 10 minutes of incubation, either because of desensitization or as a result of inhibition of adenylyl cyclase by 5-HT receptors in the same tissue. In guinea pig cerebral cortex slices, 5-HT had no effect on cAMP, although it significantly enhanced the stimulation of cAMP production by adenosine (Huang et al., 1970; Schultz and Daly, 1973) or by depolarizing the slices with potassium (Shimizu et al., 1970). 5-HT (10^{-4} M) increased cAMP accumulation in porcine hypothalamic slices but not in porcine cerebral cortex or anterior pituitary tissue (Sato et al., 1974). Small (relative to norepinephrine and/or histamine) stimulation of cAMP accumulation by 5-HT was also observed in slices from human cerebral cortex (Tsang and Lal, 1977, 1978), squirrel monkey cortex (Skolnick et al., 1973), and bovine superior cervical ganglion (Roch and Kalix, 1974).

Von Hungen and Roberts (1973) developed a system for measuring adenylyl cyclase activity in particulate preparations from rat brain, and reported that 5-HT did not stimulate AC activity in adult rats. 5-HT did stimulate adenylyl cyclase activity in particulate preparations from immature rats (Von Hungen et al., 1974; 1975). The stimulatory effect of 5-HT is the most marked in infant rat collicular membranes. The SNR in this preparation (approximately 20) is sufficiently high to allow pharmacological analysis of the receptor. Enjalbert et al. (1978a,b) investigated the regional distribution of the 5-HT receptor positively linked to AC in collicular, hypothalamic and spinal cord homogenates with tissue from

newborn rats and adult guinea pigs. 5-HT has a similar EC_{50} value (approximately 1 µM) in all tissues tested, and is distinct from dopamine and norepinephrine-stimulated adenylyl cyclase activities. Electrolytic lesioning of the serotonergic input did not alter the characteristics of the 5-HT effect, suggesting that these 5-HT receptors are postsynaptic and not regulated by exposure to 5-HT. Nelson et al. (1980 a,b) demonstrated that the regional distribution and rank order of affinities of this receptor did not correspond to the $5\text{-}HT_1$ binding site. The most potent antagonist for the rat collicular receptor appears to be spiperone (Nelson et al., 1980 b), with a K_i of approximately 200 nM (calculated from the reported IC_{50} value). The low affinity of spiperone and methiothepin in antagonizing the infant rat coliculli receptor demonstrates that it does not resemble either the $5\text{-}HT_1$ or $5\text{-}HT_2$ class of receptor. The low affinity of BOL for the rat colliculi receptor (Von Hungen et al.,1975) and the low affinity of spiperone for the liver fluke receptor indicates that these two receptors are not congruent.

In membranes prepared from NCB-20 neuroblastoma cells, 5-HT stimulates AC activity with an EC_{50} of 0.5 µM (MacDermot et al., 1979). NCB-20 cells are derived from the fusion of mouse neuroblastoma and hamster brain cells. Standard errors were not reported for the measurements, but based on the size of the error bars representing the standard deviations of the data, the SEM can be estimated, and the SNR for this system is high (approximately 40), making it suitable for pharmacological analysis of the receptors that mediate the response. Mianserin and fluphenazine are potent antagonists with K_b values of approximately 50 nM. LSD, methysergide and metergoline are all partial agonists at this receptor, as they are at the liver fluke 5-HT receptor (see above), and LSD has nearly identical EC_{50} values in both preparations,

approximately 10 nM. The cAMP content of intact NCB-20 cells was measured by Berry-Kravis and Dawson (1983), and they reported a K_i value of 22 µM for spiperone in inhibiting 5-HT-stimulated cAMP formation. However, measurements of cAMP levels in intact cells, not surprisingly, exhibited a much lower SNR than in membranes, approximately 6 to 7 based on the results shown in Berry-Kravis and Dawson, 1985a. These authors classify the response as mediated by 5-HT_1 receptors, but the relevant drugs for that classification (5-CT, methiothepin) have not yet been tested. The low affinity of spiperone at the NCB-20 receptor suggests that it is different from infant rat collicular and 5-HT_{1A} receptors. 5-HT stimulates synthesis of gangliosides in NCB-20 cells over the same concentration range that it stimulates cAMP formation (McLawhon et al., 1981), suggesting a possible role for cAMP production in these cells. Incubation of NCB-20 cells with gangliosides decreases the EC_{50} of 5-HT in stimulating cAMP formation by as much as 20-fold, although whether the effect results from specific incorporation of the gangliosides into the receptor or from a change in membrane fluidity has not been ascertained (Berry-Kravis and Dawson, 1985). Opiates reduce the cAMP accumulation in response to 5-HT, and effects of opiates desensitize after 12 to 24 hours, resulting in cells that are supersensitive to 5-HT as evidenced by a reduction in the EC_{50} value and increase in the maximal rate of activation by 5-HT (Berry-Kravis and Dawson, 1985). Incubation of supersensitive NCB-20 cells with gangliosides does not further sensitize the cells to the effects of 5-HT.

5-HT stimulates AC activity in homogenates from adult rat (Barbaccia et al., 1983) and guinea pig (Shenker et al., 1983) hippocampi. Analysis of the receptor mediating the response in guinea pig homogenates demonstrated the existence of two distinct populations of 5-HT receptors, a high affinity

receptor corresponding to the 5-HT_{1A} binding site, and a lower affinity receptor that may correspond to the infant rat collicular receptor (see discussion). Results with rat hippocampal membranes indicate that 5-HT_{1A}-mediated stimulation of AC is present in rat hipocampus as well (Markstein et al., 1986), although the SNR is low in this tissue, (approximately 7),and the rank order of the agonists differs considerably from the order obtained by measuring inhibition of adenylyl cyclase activity (see discussion). Further analysis of the guinea pig and rat hippocampal 5-HT_{1A} receptor suggests that this receptor may be preferentially negatively coupled to AC and that the physiological effects of this receptor require coupling to the inhibitory regulatory protein, G_i (see discussion). 5-HT stimulates AC activity in membranes from primary cell cultures of mouse striatal neurons (Premont et al., 1983) and mesencephalic neurons (Chneiweiss et al., 1984). Both responses are anatagonized by fluphenazine, with a K_b of approximately 1 µM based on the shift of the concentration-response curve to 5-HT, similar to its value for the infant rat colliculi receptor and for the hippocampal receptor (see results), but the antagonism is noncompetitive, and definitive conclusions cannot be made as to the homology among these receptors. 5-HT also stimulates cAMP formation in murine striatal and cerebral cortical cells (Weiss et al.,1986). Fillion et al., (1979;1980) reported the presence of high (EC_{50} = 1 to 3 nM) and low (EC_{50} = 1 µM) affinity 5-HT receptors coupled to AC in horse and rat striatal membrane preparations, but the low SNR of these systems (2-3 for the high affinity receptors, 5 -6 for the low affinity receptors) suggests that, although the effect of 5-HT may be significant, it is difficult to accurately determine EC_{50} values in these preparations. Kainic acid lesioning of intrinsic neurons in rat striatum abolished the stimulation by the receptor with nanomolar

affinity for 5-HT. In addition to these cases, 5-HT-stimulated AC activity has been measured in homogenates from guinea pig basal ganglia (Rasenick et al., 1986), whole rat brain (Pagel et al., 1976), and monkey anterior limbic cortex (Ahn and Makman, 1978a,b).

In conclusion, a 5-HT receptor positively coupled to AC in mammalian brain has been characterized in membranes from infant rat colliculi, and a homologous receptor may be present in guinea pig hippocampal membranes (discussion). The 5-HT receptor on NCB-20 cells appears to differ pharmacologically from the collicular receptor, suggesting that a second, distinct 5-HT receptor may be positively coupled to AC in mammalian brain. Tentatively, the 5-HT receptor mediating the increase in AC activity in murine striatal and mesencephalic neuronal membranes is of the infant rat collicular class.

I.4 Decreases in cAMP mediated by 5-HT

Historically, most experiments testing the effects of drugs on cAMP levels have been designed to test stimulation by these drugs. Therefore, evidence for 5-HT mediated inhibition of cAMP accumulation has been indirect; the result of testing the effect of 5-HT on the stimulation produced by other factors, usually norepinephrine or epinephrine. Norepinephrine and epinephrine inhibit AC activity at α_2-adrenergic receptors, and therefore experiments with these drugs and 5-HT in combination may have obscured inhibitory efects of 5-HT. Most experimental protocols were designed to label the precursor pool of ATP in a tissue and then measure the amount of labeled cAMP in response to an agonist. Since conditions were chosen to

minimize use of radioactive materials, the basal activity was chosen to be as low as could be measured over the noise of the system. Therefore, these experiments were inherently unable to detect inhibitory effects of agonists, since such an effect would be lost in the noise of the system. In spite of these considerable experimental limitations, there are many indications in the literature of 5-HT receptors that decrease cAMP levels in intact tissues or membranes.

Davoren and Sutherland (1963), in one of the earliest papers to report on epinephrine-stimulated cAMP content of intact cells, reported that 5-HT partially inhibited the epinephrine-induced increase in cAMP content of whole pigeon erythrocytes. These findings were later corroborated by Sheppard and Burghardt (1970, 1971) with rat erythrocytes. Campbell and Siddle (1977) investigated the pharmacological characteristics of this inhibition, and reported that 5-methoxytryptamine was equipotent with 5-HT in blocking cAMP accumulation by epinephrine in pigeon erythrocytes, as it is in inhibiting forskoln-stimulated AC activity (see results).

5-HT partially antagonizes norepinephrine-stimulated cAMP production in vitro in hypothalamic slices (Palmer et al.,1973). Administration of the 5-HT releasing drug p-chloroamphetamine (PCA) completely antagonizes norepinephrine-induced increases in cAMP content of rat hypothalamic slices and partially decreases basal cAMP levels (Palmer et al., 1972). Imipramine, a tertiary amine, also blocked norepinephrine stimulation, but the secondary amine protryptiline did not. Tertiary amines effectively block 5-HT neuronal uptake mechanisms but the corresponding secondary amines are much less active, suggesting that the effect of imipramine resulted from its 5-HT uptake blocking properties. However, lesioning of the raphe input to the cortex and hippocampus or blocking 5-HT uptake with

fluoxetine did not alter the characteristics of norepinephrine-stimulated cAMP formation, thereby refuting a role for modulation by 5-HT of ß-adrenergic stimulated cAMP formation (Mishra et al.1978,1981). In contrast to the results of Mishra et al., Stockmeier et al. (1985) measured an increase in ß-adrenergic stimulated cAMP formation in slices of rat cerebral cortex and hippocampus after lesioning of the raphe. The increase in ß-adrenergic stimulated cAMP formation was interpreted as an increase in the number of ß-receptors in the slice. An alternative explanation is that the lesion removed an endogenous inhibitor from the slice, possibly 5-HT.

Weiss and Costa (1968) measured catecholamine-stimulated adenylyl cyclase activity in pineal gland homogenates. 5-HT inhibited basal activity and completely blocked the stimulation of adenylyl cyclase by norepinephrine. In cultured rat astroglial cells, 5-HT and melatonin inhibited isoproterenol-stimulated cAMP formation, and melatonin was more potent than 5-HT, suggesting that the effect may be mediated by melatonin rather than 5-HT receptors (Vacas et al., 1984). Since melatonin is produced in the pineal gland, the effect measured by Weiss and Costa could have resulted from an interaction of 5-HT at melatonin receptors.

Inhibition of adenylyl cyclase activity by 5-HT in lysates of human platelets has been reported (Zieve and Greenough, 1969), but other investigators have reported that 5-HT does not affect cAMP levels in human platelets (Agarwal and Steiner,1976; Affolter et al., 1984). Agarwal and Steiner reported that 5-HT did stimulate cGMP production in platelets.

5-HT increases intestinal motility in isolated intestinal segments of dogs (Grubb and Burks, 1974, 1975) as monitored by an increase in intraluminal pressure. The concentration-response curve to 5-HT was shifted to the right by agents that increase cAMP accumulation

(theophylline, norepinephrine and PGE_1). These agents did not block the response of the segments to bethanecol or DMPP, cholinergic agonists. Therefore, cAMP specifically blocked the response of the system to 5-HT, suggesting to the authors that the direct intestinal smooth muscle excitatory effects of 5-HT occurred as a result of lowered intracellular levels of cAMP. 5-HT receptors also have complicated effects presynaptically in intestinal tissue, since M receptors increase release of acetylcholine and 5-HT_{1A} receptors inhibit release in guinea pig ileum (see section I.2). If the direct contractile response of 5-HT on intestinal tissue is mediated by a receptor negatively coupled to AC, then the "D" receptor response studied by Gaddum and Picarelli (1957) may be mediated at least partly by 5-HT_{1A} receptors.

Inhibition of adenylyl cyclase activity by 5-HT receptors has been characterized in guinea pig and rat hippocampal membranes (see results) and in intact murine striatal and cortical cells (Weiss et al., 1986). The inhibition is mediated by 5-HT_{1A} receptors.

In conclusion, in various tissues (erythrocytes, intestinal smooth muscle, hypothalamic slices, pineal gland homogenates, brain stem slices, platelets), in both intact preparations and homogenates, and in vitro preparations or after in vivo release, 5-HT blocks norepinephrine-stimulated AC activity or the effects of norepinephrine-stimulated AC activity. The presence of the 5-HT receptor negatively linked to AC may have masked the presence of stimulatory receptors and possibly accounts for discrepancies among data obtained on 5-HT stimulated AC activity.

I.5 Potential roles for cAMP in neurons

The specific pathways between activation of adenylyl cyclase and physiological responses has been elucidated in few cases. The best example is glycogen metabolism in liver, where an enzymatic cascade amplifies the stimulus from agonist binding to activation of phosphorylase more than a thousand-fold (Stryer, 1981). Glycogenolysis is induced by 5-HT in murine cerebral cortex slices (Quach et al., 1982), and therefore carbohydrate metabolism is a potential role for 5-HT-stimulated cAMP formation. Two other neuronal systems responsive to 5-HT and cAMP are discussed in this section in order to illustrate how 5-HT might mediate changes in cell function by its effect on cAMP.

Electrically-evoked release of 5-HT is apparently dependent upon Ca^{++} and facilitated by cAMP (Schoffelmeer et al., 1985). Because of its location and its modulation by both Ca^{++} and cAMP, synapsin is a candidate as a phosphoprotein mediating transmitter release (Nestler and Greengard, 1983). Synapsin (protein I) is phosphorylated at three distinct sites, two serine residues that are phosphorylated by Ca/Calmodulin protein kinase I and another serine residue phosphorylated by both cAMP-dependent protein kinase and Ca^{++}/calmodulin protein kinase II. Synapsin is associated with all synaptic vesicles in all nerve terminals (Nestler and Greengard, 1983). It binds with high affinity to vesicles (Schiebler et al., 1986) and microtubules (Baines and Bennett, 1986) and, in immunocytochemical studies, is found associated with neuronal cytoskeletons (Goldenring et al., 1986). Therefore, sysnapsin may link vesicles to the cytoskeleton. Phosphorylation of synapsin by Ca^{++}/calmodulin, but not by cAMP-dependent protein kinase, reduces the affinity of synapsin for vesicles (Schiebler et al., 1986). From these results, a possible biochemical model for

transmitter release can be formulated: synapsin, in its dephosphorylated form, anchors vesicles to the neuronal cytoskeleton. Depolarization of the neuron increases Ca^{++} in the cell, causing phosphorylation of synapsin and decreasing its affinity for the cytoskeleton, allowing vesicles to fuse with the plasma membrane and make transmitter release possible. The role of cAMP-dependent protein kinase is not yet clear, but possibly it facilitates the actions of Ca^{++}. In glutaminergic neurons presynaptic to facial motor neurons, synapsin is found to be phosphorylated by 5-HT and the effect is partially antagonized by high concentrations (10 μM) of mianserin and metergoline (Dolphin et al., 1980; Dolphin and Greengard, 1981a,b). The effect of 5-HT is enhanced by the phosphodiesterase inhibitor IBMX, suggesting that cAMP may have a role in the process, but is also dependent upon Ca^{++}, suggesting that cAMP may modulate the activity of Ca^{++} in this system. Interestingly, S1 receptors (see above) modulate the excitatory postsynaptic effects effects of glutamate on facial motor neurons (McCall and Aghajanian, 1979). 5-HT may have a dual action in this tissue, a presynaptic facilitation of glutamate release and a postsynaptic facilitation of the excitatory effects of glutamate (Nestler and Greengard, 1983).

Changes in conductances of ions in response to changes in cAMP have been difficult to demonstrate convincingly. Responses that were claimed to be mediated by cAMP, such as depolarization of Purkinje cells in the cerebellum or hyperolarization of cervical ganglion neurons, were later challenged by investigators who attributed the responses to extracellular effects of the cAMP analogues (reviewed in Phillis, 1977). Invertebrates contain much simpler nervous sytems with larger neurons, and these properties have been exploited to study receptor transduction systems in

neurons. Perhaps the most thoroughly studied receptor transduction system is that of the sensory neurons of *Aplysia* ganglia (reviewed in Kandel and Schwartz, 1982). This sytem serves as a molecular model for learning behavior. Short-term sensitization of the *Aplysia* to a gill-withdrawal stimulus results in increased 5-HT released from modulatory neurons that synapse with sensory neurons. 5-HT elicits an increase in cAMP in the presynaptic terminal, facilitating transmitter release. The facilitation is mediated by the effects of cAMP which causes more Ca^{++} to enter the cell during depolarization by broadening the action potential. Prolongation of the action potential by cAMP is mediated by depressing a K^+ current (Klein and Kandel, 1980). The actions of 5-HT on this K^+ conductance, designated I_{Ks}, are mimicked by intracellular injection of cAMP-dependent protein kinase and blocked by cAMP-dependent protein kinase blockers, suggesting that cAMP phosphorylates the channel or a protein that modulates the channel (Castellucci et al., 1980). In these neurons, therefore, the interactions of cAMP, Ca^{++} and K^+ channels can be observed, and the learning behavior of the *Aplysia* (the withdrawal reflex) can be studied as a function of these different effector systems.

I.6. Mechanistic aspects of adenylyl cyclase activation

The fluid mosaic model of the cell membrane (Singer and Nicolson, 1972) radically altered receptor transduction theory by suggesting that proteins diffuse through the cell membrane. Previously, receptors were thought to be inextricably associated with effectors, like nicotinic receptors and Na^+ ion channels. Cuatrecasas (1974) suggested that receptors and effectors

(such as the catalytic moiety of adenylyl cyclase) were separate, mobile entitities that interacted within the cell membrane. The hypothesis was strengthened by the studies of Orly and Schramm (1976) who demonstrated that receptors from one cell could migrate and interact with the adenylyl cyclase of a different cell upon cell fusion.

A second important advance in understanding adenylyl cyclase systems was the discovery that GTP enhanced the effect of receptor agonists in stimulating adenylyl cyclase activity (Rodbell et al., 1971). The GTP-binding activity could be resolved from the receptor (Pfeuffer and Helmreich, 1975), demonstrating that the GTP-binding moiety and the receptor were distinct entities. These findings identify the proteins involved in signal transduction in adenylyl cyclase systems as the receptor and a GTP-binding protein. In addition to GTP, Mg^{++} may also be required for activation (Birnbaumer et al, 1969). Receptors can tap the potential chemical energy of GTP and Mg^{++} in the cell and not simply the energy obtained from the agonist binding to the receptor, much like nicontinic receptors tap the potential electrical energy stored in the separation of charge across membranes.

Tolkovsky and Levitzki (1978) modeled the kinetic behavior of AC activation in membranes from turkey erythrocytes and formulated the idea of "collision coupling" whereby the drug (D) binds to the receptor (R) and, in turn, the DR complex activates the effector (E) in such a way that the DRE complex does not accumulate. This model satisfies the observations that the rate of response (in the presence of Gpp(NH)p) is linearly proportional to receptor concentration, but that the maximal response is independent of receptor concentration. These data support the idea that DR acts catalytically, since reducing receptor number does not reduce the maximal

rate of activation of of AC (i.e. one receptor can activate many effectors). In the presence of GTP, the rate of GTPase activity would presumably alter the relationship between concentration of R and maximal rate of activation. In the kinetic collision coupling model, efficacy is related to the ratio of k_{on} to k_{off}. The parameter k_{on} is the rate of flux for the formation of DRE from DR and E, and k_{off} is the net flux towards deactivation of the DRE complex, either by GTP hydrolysis or, in the case of nonhydrolyzable analogs of GTP, the dissociation of guanyl nucleotides from E.

A second important model emerged from studies with guanyl-nucleotides. Incubation of solubilized ß-adrenergic receptors with agonists, but not with anatgonists, resulted in an apparent increase in the size of the receptor (Limbird and Lefkowitz, 1978) and Gpp(NH)p reversed the effect. The increase in size of the ß-adrenergic receptor was associated with an increase in the labeling of a GTP-binding protein (N or G protein) by ADP with cholera toxin (Limbird et al., 1980). In binding studies, GTP reduced the affinity of agonists (but not antagonists) for their receptors (Maguire et al., 1976). De Lean et al. (1980) proposed the ternary complex model to explain these effects of guanyl nucleotides. The model assumes two states of the receptor: a high affinity state where R is bound to G and a low affinity state where R is separate from G. Agonists and Mg^{++} promote the coupled, high affinity state and guanyl nucleotides promote the uncoupled, low affinity state. This steady state model differs from the kinetic collision coupling model in that the DRG complex accumulates to a measurable degree. However, this complex accumulates only in the absence of GTP, therefore the collision coupling model may be appropriate under conditions where GTP is present (i.e. in vivo). In the ternary complex model, efficacy is related to a drugs ability to stabilize the RG state. At steady

state, this model of efficacy can be identical to the ternary complex model, since by increasing the rate of coupling of R to G, their would be a higher level of the high affinity state, RG, measured at any time t, keeping the k_{off} parameter constant.

Only in artificially reconstituted preparations can all the components of adenylyl cyclase systems be known and quantitated. The components of the adenylyl cyclase system include receptors , G proteins and the enzyme itself, the catalytic unit (C).

The catalytic unit, C, has been resolved from the other components of the system (Strittmatter and Neer, 1980) and purified on forskolin-affinity columns (Smigel,1986). The resolved catalytic unit has an approximate molecular weight of 120,000. It is unable to utilize Mg^{++} •ATP as a substrate but does utilize Mn^{++}•ATP. Addition of forskolin, G_s or calmodulin/Ca^{++} activates C such that it uses Mg^{++}•ATP as a substrate. Injection of forskolin into Xenopus oocytes does not stimulate cAMP formation, although forskolin applied in the extracellular medium does (Schorderet-Slatkine and Baulieu, 1982). This suggests that the catalytic unit itself may have an extracellular site. The catalytic unit has the properties of an integral membrane protein, making it conceivable that it possesses both an intra- and extracellular site (Ross et al., 1983). The extent of activation of the catalytic unit is increased if G_s is added together with forskolin, indicating forskolin may also have a stabilizing effect on G_s-C interactions (Smigel, 1986). Despite ignorance as to its mode of action, forskolin is a useful tool for discovering and measuring the effects of receptors that inhibit cAMP formation or AC activity.

During the course of isolating components of the adenylyl cyclase system, it became apparent that more than one G protein exists. To date, at

least five G proteins have been isolated, and the cDNA for as many as eight proteins has been discovered (Bourne, 1986). G_s, the G protein positively coupled to the catalytic unit of adenylyl cyclase, may exist in two different forms based on differences in the sequences of cloned G_s (Robishaw et al., 1986). G_i is negatively coupled to adenylyl cyclase and G_t (transducin) is coupled to cGMP phosphodiesterase (Roof et al., 1985). The functions of G_o and G_p are unknown. These proteins were isolated from brain (Florio and Sternweis, 1985) and placental (Evans et al., 1986) tissue, respectively. In addition to these G proteins, other G proteins are postulated to exist based on the sensitivity of responses to guanyl nucleotides. Calcium gating in mast cells is modulated by intracellular injection of guanyl nucleotides (Gomperts, 1983) and hormone-stimulated phosphatidylinositol turnover in membranes from GH_3 cells is enhanced by GTP (Straub and Gershengorn, 1986). Muscarinic receptors coupled to potassium channels in heart may be coupled to a GTP-binding protein (Pfaffinger et al., 1985). Therefore, many cAMP-independent cellular responses involve GTP-binding regulatory proteins. Related to G proteins are other GTP-binding proteins important to cell biology, including elongation factors (proteins that modulate protein transcription) and oncogene products. Elongation factors, such as bacterial EF-TU, have been crystallized, revealing information as to the nature of the GTP-binding sites of these proteins and the regions that interact with effectors and receptors (Bourne, 1986). The relationship among oncogenes and G proteins is not yet clear, but incorporation of ras oncogene stimulates phosphatidylinositol turnover in fibroblasts, suggesting it may be an G protein mediating activation of phospholipase C (Fleischman et al., 1986).

G proteins are similar in certain respects. They all bind GTP, are

GTPases and they are composed of at least three subunits: α, β, and γ. The β and γ are apparently identical for all of the G proteins both in terms of their molecular weights and immunoreactivity (Roof et al., 1985). The α subunits are different in weight, immunoreactivity and function. The mechanisms of activation of G proteins and the consequent activation of effectors by G proteins are not well understood. G_s proteins dissociate upon incubation with nonhydrolyzable guanyl nucleotides and Mg^{++} , suggesting that liberation of the free α_s subunit may be essential, but GTP itself does not cause subunit dissociation, raising the possibility that dissociation is not required for activation of G proteins (Birnbaumer et al., 1985).

Receptors that couple to G proteins have been purified and reconstituted in functional cells, including β_2-adrenergic receptors from rodent lungs (Benovic et al., 1984), and α_2-adrenergic receptors from human platelets (Cerione et al., 1986). The β_2-adrenergic receptor, for example, is a single polypeptide of molecular weight 64,000. In addition to hormone receptors, many other types of receptors may transduce information through G proteins, including immunoglobulins (Gomperts and Fewtrell, 1985), rhodopsin (Stryer et al., 1981) and olfactory receptors (Pace and Lancet, 1986). In retinal cells, rhodopsin serves as a receptor for photons and, in turn, interacts with transducin. The activation of transducin activates a cGMP phosphodiesterase and reduces the amount of cGMP present in the cell. The reduction in cGMP-dependent protein kinase activity may open a Na^+ channel, hyperpolarizing the cell. Antigens bind to IgE on mast cell membranes and mobilize Ca^{++}, causing exocytosis, and the effect of antigens is modulated by the presence of guanyl nucleotides.

Effectors coupled to G proteins are often enzymes, including adenylyl cyclase, cGMP phoshodiesterase and, possibly, phospholipase C, the enzyme

that liberates inositol phosphates and diacylglycerol from membranes and mobilizes intracellular Ca^{++}. The effectors may also be ion channels, such as potassium channels in heart muscle or, possibly, Ca^{++} channels (Gill et al., 1986). These effectors produce changes in second messengers inside the cell that regulate the activity of kinases in the cell. Kinases control the level of phosphorylation of phosphoproteins, which in turn mediate physiological responses. The G protein system may be an integrated system responding not only to hormones and neurotransmitters but also to antigens and other sensory information, such as light and odors. The G protein system modifies electrical and chemical properties of cells and affects all aspects of cell physiology, including gene expression, cytoskeletal structure, membrane potential, cell proliferation, cell secretion, cell contraction, exocytosis, and metabolism. A somewhat speculative diagram of the pathway of events from drug to physiological response is shown in fig. 1.

Within the transduction pathways shown in fig. 1, the path from application of drug to physiological response is often not a simple vertical one, but involves cross-activation of parallel pathways as well. For example, G proteins can influence the activity of other G proteins in the same cell: activation of any G protein should inhibit the activity of all others in the same cell by releasing $\beta\gamma$ subunits; activated G proteins may exchange GTP with other G proteins, activating them also (Hatta et al., 1986); and stimulation of adenylyl cyclase by G_s is much greater in terms of fold stimulation over basal if G_i is included in the reconstituted cells (Cerione, 1986). Effectors can regulate G proteins (stimulation of protein kinase C may inhibit G_i; Katada et al., 1985) or other effectors (Ca^{++} stimulates adenylyl cyclase; Rasmussen and Barrett, 1984). Receptors and

ion channels are regulated by effectors: purified nicotinic receptors are phosphorylated by both cAMP-dependent protein kinase and protein kinase C, and K^+ channels in *Aplysia* neurons are modulated by cAMP-dependent protein kinase (Browning et al., 1985). Drugs may produce a single effect by different receptors: epinephrine stimulates glycogenolysis by both ß-adrenergic/ cAMP and α_2- adrenergic/ Ca^{++} pathways in rat hepatocytes (Studer and Borle, 1982). Receptors may interact with multiple G proteins to produce an effect on a cell: angiotensin II couples multiple G proteins in rat hepatocytes (Johnson et al., 1986). A single G protein may mediate multiple responses in a cell: G_i may mediate Ca^{++} mobilization in neutrophil membranes (Okajima et al., 1985). Therefore, all G protein systems are interdependent, and the integrated effect of receptor stimulation on a cell may involve numerous transduction pathways. The effect of receptor stimulation may be state dependent, in other words the effects may depend on the preexisting level of activation of G proteins and phoshorylation of phosphoproteins within the cell. An interesting outcome of the interaction among G proteins and receptors is that drugs may affect sensory or immunological responses in unforeseen ways, perhaps modulating acuity of vision or influencing allergic reactions under certain circumstances.

GTP-binding proteins and their effectors are the targets of many bacterial toxins. These toxins have been useful in identifying purified G proteins. Pertussis toxin ADP-ribosylates G_i, and other G proteins as well, interfering with receptor coupling to G_i (Katada and Ui, 1982). In some cells (mast cells, e.g.) , PT abolishes receptor-mediated phosphatidylinostol turnover as well as inhibition of adenylyl cyclase (Nakamura and Ui, 1985), but in other cells phosphatidylinositol turnover is insensitive to PT treatment, for example angiotensin II-stimulated phosphatidylinositol

turnover in hepatocytes (Johnson et al., 1986). Cholera toxin ADP-ribosylates G_s and blocks the GTPase activity of this protein, permanently activating it (Van Heyningen, 1986). Diptheria toxin ADP-ribosylates elongation factor 2 and disrupts protein synthesis (Van Heyningen, 1986). A component of anthrax toxin may be an adenylyl cyclase which, when injected into eucaryotic cells, is stimulated by calmodulin to cause an uncontrolled rise in cAMP levels (Leppla, 1984). Therefore, pertussis, cholera and anthrax toxins all raise cAMP levels through distinct mechanisms.

Reconstituted cells and cell lines lacking components of the adenylyl cyclase system have been used to probe the mechanism of action of G_i. Originally, it was believed G_i exerted its inhibitory action by dissociating into subunits, causing liberation of $\beta\gamma$ subunits and driving the equilibrium of G_s dissociaion to the associated, inactive form of the protein (Katada et al., 1984). However, in cyc⁻ cells which lack G_s, the α_i subunit was shown to be sufficient for inhibiting adenylyl cyclase activity (Roof et al., 1985). In reconstituted cells, $\beta\gamma$ inhibits forskolin-stimulated adenylyl cyclase activity only in the presence of G_s (Cerione et al., 1986), suggesting that inhibition results from blocking stimulated activity, either by G_s in the case of these reconstituted cells or, presumably, a different stimulator of AC activity in the cyc⁻ cells. In purified preparations of C, the $\beta\gamma$ subunit did inhibit basal activity of C, but the inhibiton was attributed to a small contamination of the enzyme with α_s (Smigel, 1986). In partially purified preparations of catalytic unit, Katada et al. (1986) measured a direct inhibition by $\beta\gamma$ subunits in the absence of G_s, but the extent of inhibition was more pronounced after stimulation by G_s. The α_i subunit inhibited only G_s-stimulated activity. On the basis of these data, the authors proposed that $\beta\gamma$ directly inhibits the catalytic unit and that α_i subunits may

compete for and block the site of stimulation of α_s on the catalytic unit. In conclusion, it seems that in all cases $\beta\gamma$ subunits are sufficient for inhibiting G_s-stimulated AC activity, but the role of the α subunits is still questionable. An important result from all of these reports is that, if the $\beta\gamma$ units are solely responsible for inhibition, all G proteins that dissociate upon activation should inhibit AC activity to the same extent, and G_i is no more specific for inhibition of adenylyl cyclase than any other G protein, including G_s. This would mean that the specific role of G_i has yet to be discovered. These results would imply that activation of any G protein should be sufficient to inhibit activity of all others, since the $\beta\gamma$ subunit is common to all G proteins.

The observation that denervated tissues develop increased sensitivity to hormones is an old one, about as old as the concept of receptors itself (reviewed in Trendelenburg, 1963). Isolation of the components of the AC system allows elucidation of the membrane events that contribute to phenomena such as denevation supersensitivity and desensitization. Studies with intact cells show that exposure of β-adrenergic receptors to isoproterenol results in a brief but many-fold increase in cAMP accumulation, resembling a spike on a time scale, followed by a reduction in cAMP accumulation that is more rapid than the decrease in the number of receptor-binding sites (Su et al., 1980). The transient nature of the increase probably complicates pharmacological analysis of AC-coupled receptors in intact cells. In addition to receptor desensitization, other factors contribute to the decay of the cAMP signal (as mentioned in section I.3). Two mechanisms of receptor desensitization at the membrane level have been reported for β-adrenergic receptors. β-adrenergic receptors from rat lung become sequestered in a light vesicle fraction and thereby

preveted from interacting with the AC system, although they retain their capacity to stimulate AC if reconstituted into cells with functional G_s (Strasser et al., 1985). This sequestering may be a prelude to receptor internalization. A second mechanism has been demonstrated in turkey erythrocytes. In these cells, ß-adrenergic receptors are phosphorylated by an enzyme designated BARK (for ß-adrenergic receptor kinase) and lose their capacity to stimulate G_s (Benovic et al., 1986). An interesting aspect of this phenomenon is that BARK phosphorylates the ß-adrenergic receptor independently of cAMP, although whether receptor activation stimulates BARK or simply exposes a site on the receptor that is a substrate for the kinase, is not known (Strasser et al., 1986).

I.7 Relationship between pharmacological theory and receptor-transduction studies.

Pharmacological theories of drug action attempt to relate the magnitude of a physiological response with the concentration of drug at the receptor. After the initial postulate of receptive substances (Langley, 1905; Ehrlich, 1913, as reviewed in Ruffolo, 1982), Clark (1926) quantitated the relationship between response and agonist concentration by assuming that the response reflected the binding of an agonist to the receptor through the law of mass action. This theory was not sufficient to account for differences in maximal responses in the same tissue by different agonists, therefore Ariens (1954) introduced a new constant into the equation, intrinsic activity, which scaled the maximal response obtainable by partial

agonists relative to full agonists. Stephenson and Furchgott realized that full responses could be obtained with less than 100% occupancy of receptors. Stephenson (1956) introduced the term efficacy (e) which related receptor occupancy to the stimulus produced by the occupied receptor in a given tissue. Stimulus was related to response by an undefined property of the tissue. Furchgott (1966) expanded on the idea of efficacy by realizing that efficacy contained within it a parameter equal to the total receptor concentration in the tissue, therefore he divided efficacy by $[R]_{tot}$ and called it intrinsic efficacy, ϵ Intrinsic efficacy is independent of the tissue and is a property of the drug itself.

Both Tolkovsky (1983) and Black and Leff (1983) gave a physical meaning to ϵ by assuming that the generation of a response was governed by the equilibrium between occupied receptors (DR) effectors, E. The nature of agonism is to alter the equilibrium between DR and E. These models were influenced by the ternary complex and collision coupling models reviewed in section I.6. In the Black and Leff model,e is redefined as the ratio of R_{tot} to a new parameter, K_E. The parameter K_E is operationally defined as the concentation of DR that produces a half-maximal response in a system. This parameter is assumed to be proportional to the affinity of DR complexes for effectors.

Do current concepts of receptor transduction mechanisms support pharmacological models? The mechanism of activation of G proteins is still not understood, but the activation is coincident with an increase in binding of guanyl nucleotides to the G protein. In reconstituted phospholipid vesicles, Asano and Ross (1984) showed that receptor agonists increase the rate of GTPγS binding to G_s, that the increase in GTPγS binding parallels the activation of adenylyl cyclase, and that the increase in GTPγS binding

was saturable. These data suggest that the function of the drug-receptor complex is to activate G_s. The activity of the agonist can be measured in the absence of functional adenylyl cyclase (i.e. by measuring the increase in the apparent rate constant of GTPγS to G_s), demonstrating that the interaction of R with G_s is sufficient to transduce receptor information to G_s. The effect of receptor stimulation can be measured by the activation of the G protein. Rough calculations from the data suggested to the authors that one liganded receptor activated up to eight G_s molecules, and the K_d of isoproterenol binding was 50-fold higher than its EC_{50} for stimulating GTPγS binding, suggesting a catalytic role for the receptors and supporting the collision-coupling model of AC activation. According to the Asano and Ross model, DR dissociates from G and activates other G proteins, and the activated G_s protein presumably dissociates to α_s•C, the active form of adenylyl cyclase. However, based on the resistance of hormone-stimulated AC activity (but not activated G_s-stimulated activity) to inhibition by $\beta\gamma$ subunits, Cerione et al. (1986) suggested that the active species that stimulates AC activity is DR•α_s•C. (or DR•G_s•C), not α_s•C as in the Asano and Ross model. Turnoff of G_s activation in the Cerione model occurs at the GTPase step, causing dissociation of DR and α_s, and consequently D from R. This model is not consistent with collision coupling or the catalytic mode of receptor coupling, since R is forms a complex with the effector when the effector is active, therefore the maximal response must be proportional to receptor concentration if the number of receptors is smaller than the number of G_s molecules. The basic difference in the Asano model and the Cerione model is that, in the Asano model, the receptors act catalytically, small numbers of receptors activate a larger number of G proteins, and spare receptor phenomenon can occur even if the number of receptors were

lower than the number of effectors in the system. In the Cerione et al. model, spare receptor phenomenon could occur only if the number of receptors is greater than the number of effectors in the system. In the Cerione model, the dissociation rate of the DR complex from G is unimportant, since the GTPase step determines the rate of dissociation of the DR complex from the G protein, and the GTPase activity should be independent of the DR complex. Intrinsic efficacy would be proportional to k_{on}/k_{GTP}, where k_{on} is the apparent rate constant for activation of the G protein by DR, and k_{GTP} is the rate of GTPase activity. In the Asano model, and in collision coupling, the dissociation rate of the DR complex is important, since the shorter the k_{off}, the more successful collisions can be made by the DR complex during the time it takes for GTP to be hydrolyzed. In the Asano model, intrinsic efficacy is related to the ratio between k_{on} and a factor that includes k_{GTP} and k_{off}, where k_{off} is the rate of dissociation of the DR complex from G. The difference in these two models is analogous to the difference between rate theory of receptor activation (Paton, 1961), where the agonism of a drug depends upon the number of successful collisions that can be made by an agonist molecule before receptors deactivate, and occupancy theories of drug action, where response is proportional to the number of DR complexes at equilibrium. The differences in the models are also analogous to the differences between the ternary complex and collision coupling models, since the DRG complex is measurable in the ternary complex model (as it is in the Cerione et al. model), but the DRG complex is an unmeasurable rapid intermediate in the collision coupling model. Since the receptors used by Asano and Ross were purified from turkey erythrocyte membranes, the same system used to develop the collision-coupling model, whereas the receptors used by Cerione et al. were

purified from hamster lung, the differences in the models may reflect differences in the receptors themselves.

The Black and Leff model is consistent with the results from both sets of reconstitution experiments reviewed above, and it must be applicable to any receptor-effector system if there is a saturable relationship between the DR complex and activation of the effector, since K_E is an operationally defined parameter with no mechanistic implications. In the special case of the receptor-transducer model, however, Black and Leff imply that efficacy of the drug is proportional to the affinity of the DR complex for G. This may not be strictly true, since in the Cerione model the k_{off} of the DR complex is unimportant, it is the rate of GTPase activity that determines the off rate of the DR complex, and therefore differences in efficacy of agonists would be related to the k_{on} of the complex only. In the Asano model, and in collision coupling, if the k_{off} of the DR complex is much shorter than the rate of GTPase activity (as it is in the presence of GTPγS), then the efficacy of the drug would be proportional to the affinity of the DR complex for G. Essentially, the value of the Black and Leff model is to indicate factors that influence the efficacy of a drug in a given system, allowing pharmacologists to exploit tools that modify the actions of G proteins or effectors, like cholera toxin and Ca^{++} channel agonists. With the identification of a large family of G proteins, it is apparent that models that account for agonism as a result of modifications of the interactions of receptors and G proteins should have much wider applicability than previously realized.

The ability to design systems to measure receptor-mediated responses is probably the limiting factor in receptor classification and is the basis of the science of pharmacology. Black and Leff (1983) claimed that "Hormones, and other agonists, can only be recognized by their effects on physiological,

that is intact-tissue, systems". It is difficult to understand why this assertion is made, since the effects of agonism are often measured in non-intact tissues, such as the membrane preparations used to characterize 5-HT_{1A} receptor-mediated inhibition of AC activity desribed in this dissertation. In many receptor systems, receptor-mediated responses simply modulate the activity of other inputs, and if these inputs are not present in the intact preparation, the receptor would be invisible to the pharmacologist. For example, ß-adrenergic receptors increase heart contractility by increasing cAMP and thereby modulating Ca^{++} influx. In the absence of Ca^{++}, no response would be observed in the intact tissue after receptor stimulation, although in membrane preparations these receptors would still stimulate AC activity. The Ca^{++} and cAMP messenger systems, and possibly K^{+} channel systems as well, are so inextricably linked that it may be impossible to study a physiological effect as the result of one pathway without considering cross-activation of others. In fact, as reviewed in section I.3, the primary role of 5-HT receptors that stimulate cAMP formation seems to be modulation of Ca^{++}-mediated effects, such as the release of neurotransmitters from presynaptic neurons in slices of facial motor nucleus, facilitation of *Aplysia* buccal muscle contraction, steroidogenesis in adrenal cells, and secretion from blowfly salivary glands. Therefore, if the physiological system does not include the correct input, the effects of receptor stimulation would not be measurable although receptor-stimulation would still occur.

In figure 1 , the steps from drug application to response are shown. At each step of the pathway, phenomenon such as desensitization, amplification of signal and cross-activation of parallel effector pathways obscure the relationship between drug concentration and magnitude of

physiological response, a point that has been stressed repeatedly in this review. All of these phenomena contribute to systematic differences in the physiological responses of tissues to agonists and limit analysis of receptors mediating pharmacological analysis. The Black and Leff model assumes that the response is proportional to the generation of the DRG complex, therefore monitoring the response at the level of the G protein should improve the resolution of the receptor transducing properties of the system. As the techniques for measuring functional coupling between receptors and G proteins become available, this approach should be used for pharmacological characterization of receptors. Coupling of receptors to G proteins can now be measured directly in reconstituted cells by monitoring the hydrolysis of GTP or the increase in binding of a labeled guanyl-nucleotide. It should be possible to prepare tissues in such a way as to make them permeable to guanyl nucleotides, enabling pharmacologists to monitor receptor-G protein coupling. This approach would allow the classification of receptor-mediated responses that were previously invisible to concentration-response curve analysis and would allow identification of receptors interacting with multiple effectors. It is my opinion that this tool will prove to be invaluable for pharmacologists. By monitoring the effects of receptor stimulation on the coupling of G proteins to the receptor, it should be possible to monitor the activation of the G proteins largely independently of the system itself. Some differences in systems would still be apparent, however, such as differences in membrane fluidity from experiment to experiment, so the measurements would still be of response, not stimulus, of the receptor. As techniques improve for the isolation of G proteins and receptors, it may be possible to design a system such that a unit of stimulus could actually be defined. For example, an

increase in 10 pmols of the binding of GTP to an G protein in a defined artificial membrane preparation at a particular temperature and Mg^{++} concentration would be defined as a unit of stimulus. The definition of stimulus would allow accurate determinations of the intrinsic efficacy of drugs and would also create an entirely new discipline, the recepto-dynamics of stimulus generation and stimulus-response coupling in cell membranes.

Systematic differences may explain why traditional classification schemes have largely failed for 5-HT receptor classification. Based on the criteria of similar K_b values of antagonists and rank order of agonists, a cursory examination of the literature reveals that there may be more than 10 types of 5-HT receptor present in mammalian tissues: 3 types of 5-HT_3. "D", 5-HT_2, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, two types of stimulatory receptor linked to adenylyl cyclase, a neuronal receptor in kidney, 5-HT receptors on rat fundus, basilar artery, raphe, facial motoneurons, and probably many more. Are these really different receptors or are they simply multiple manifestations of a few receptor proteins linked in different ways to effector systems? The eventual resolution of this problem will depend upon the resolution of the proteins involved and assaying them with different G proteins in order to determine the characteristics of their interactions.

I. METHODS

I. 1. ASSAY CONDITIONS

a) Total Homogenate preparation: adenylyl cyclase activity was assayed as described by Shenker et al. (1983). Male Hartley-Albino guinea pigs (400-450 g) (Perfection Breeders, Douglassville, Pa.) were killed by decapitation and the hippocampi were dissected out. The hippocampi were diluted 30:1 (volume/wet weight) and homogenized by hand (20 strokes) in a solution (buffer A) containing 300 mM sucrose, 5 mM Na_2EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT) and 20 mM Tris HCl (pH = 7.4 at 23°). The homogenate was diluted thirty-fold and centrifuged at 39,000 x g for 10 min at 4°C. The pellet from the centrifugation was resuspended in buffer A in the same volume as the homogenization. The assay was started by adding 50 µl of the resuspended total homogenate to 200 µl of the cyclase assay mix. The final concentrations of the components of the assay were: 80 mM Tris HCl (pH = 7.4 at 23°), 0.2 mM ATP (Sigma A2383), 2 mM magnesium acetate, 10 µM guanosine triphosphate (GTP), 10 µM pargyline, 0.6 mM ascorbate, 4 mM theophylline, 1 mM cAMP, 125 µg creatine phosphokinase (Sigma C3755), 5 mM phosphocreatine, approximately 1 µCi [α-P^{32}]ATP (New England Nuclear, 10-50 Ci/mmol), 80-100 µg particulate protein, 60 mM sucrose, 0.2 mM EGTA, 1 mM Na_2EDTA, 1 mM DTT and various concentrations of agonists and antagonists as specified. The assay was stopped after 135 sec by adding 100 µl of stopping solution (Salomon, 1979). [^{3}H]cAMP (20,000 cpm, New England Nuclear) was added to monitor

recovery of the [^{32}P]cAMP. The [^{32}P]cAMP was isolated and separated from [α-^{32}P]ATP by sequential chromatography on cation exchange resin (AG 50W-X4, 200-400 mesh; Bio Rad Laboratories) and alumina (ICN Pharmaceuticals) columns essentially as described by Salomon (1979). The samples were counted in a Beckman LS-9000 scintillation counter and the counts were converted into rates of adenylyl cyclase activity by subtracting the background counts, dividing the net counts by the specific activity of the [α-^{32}P]ATP, by time and by concentration of protein in the sample. The final units of activity were pmol cAMP/mg protein/min. The protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard. This tissue preparation is called total homogenate because all tissue that centrifuges at 39,000 x g is recovered.

b) Crude Membrane preparation: Guinea pigs and rats (Sprague-Dawley, 200-224 g each) were sacrificed by asphyxiation with CO_2 and the hippocampi removed. The hippocampi were diluted thirty-fold in buffer A as described above and homogenized by machine (20 strokes at medium speed). The homogenate was diluted eight-fold in buffer A and centrifuged at 500 x g at 0° for 5 minutes. The pellet from this centrifugation was discarded and the supernatant was centrifuged at 39,000 x g for 10 minutes. The pellet was resuspended in the buffer A and centrifuged again at 39,000 x g for 10 minutes. The pellet from this centrifugation was resuspended in buffer A in the same volume as the initial homogenization. The assay was started by the adding 50 µl of the membrane suspension to 200 µl of the cyclase assay mix. The final components of the assay were: 10 µl forskolin (Calbiochem), 100 mM NaCl, 10 µl GTP, 2 mM magnesium acetate, 0.2 mM ATP, 1mM cAMP, 80 mM Tris-HCl, 60 mM sucrose, 0.2 mM EGTA, 1 mM EDTA, 1 mM DTT, 1-2

μCi of [α-^{32}P]ATP, 20 - 60 μg of membrane protein, 4 mM theophylline, 10 μg creatine phosphokinase, 5 mM phosphocreatine and various concentrations of agonists and antagonists. The medium was incubated at 30° for 315 seconds and stopped by the addition of stopping solution (Salomon, 1979). The isolation of the labeled ATP was performed as described above. Forskolin was added from a 50 mM stock solution in ethanol and diluted such that the assay mixture was less than 0.02% ethanol.

II. 2 Reserpine treatment

Reserpine (Sigma) was injected i.p. (5 mg/kg body weight) into guinea pigs according to the procedure described by Shenker et al. (1983). Reserpine (5 mg) was dissolved in 125 μl of acetic acid and 250 μl of propylene glycol. The animals experienced loss of weight and behavioral manifestations of reserpinization approximately 8 hr after treatment. The animals were killed 24 hr after treatment and the hippocampi removed. Assays of adenylyl cyclase activity were done as described. Controls were treated in parallel with acetic acid and propylene glycol.

II.3 Pertussis toxin treatment

Pertussis toxin was infused (1 μg/μl; 0.5 - 3 μg; List Biologicals) into the right hippocampus of male Sprague-Dawley rats (250-300 g). as described in Clarke et al., 1987. One to three days after administration of toxin animals were anesthetized in ether and decapitated. Slices were removed (400μ) from the dorsal part of the right hippocampus and tested

for electrophysiological studies. In parallel, the remaining tissue was homogenized and used for the adenylyl cyclase assay.

II.4 5-HTP treatment

Guinea pigs were treated with 5-hydroxytryptophan (5-HTP) according to the procedure described by Luscombe et al., 1983. The peripheral decarboxylase inhibitor carbidopa (Merck, Sharp & Dohme) was dissolved in 1% carboxymethyl cellulose solution. Carbidopa (25 mg/kg body weight) was injected into control and treated animals. Treated animals received injections of 5-HTP dissolved in acidified saline at the same time as control animals received injections of acidified saline (the same solution that was used to dissolve the 5-HTP before injections). The 5-HTP and saline solutions were injected subcutaneously (80 mg/kg) and the guinea pigs were observed for signs of myoclonus as a signal that 5-HT levels were raised in vivo. Animals were sacrificed approximately one hour after the beginning of myoclonus.

II.5 Data analysis

a) Concentration-response inhibition: De Lean et al. derived the four parameter logistic equation for analysis of concentration-response curves (1978). The basal response can be estimated as a parameter rather than a perfectly known constant. The equation used to describe inhibition is:

$$R = R_i - (R_i - R_0) / [([A] / EC_{50}) + 1] \qquad (1)$$

where R is the rate of adenylyl cyclase activity (picomoles of cAMP per minute per milligram of protein) in the presence of a specified concentration of agonist, [A]; R_0 is the rate of activity in the absence of the inhibitory agonist (i.e. the rate of forskolin-stimulated activity alone); R_i is the rate after maximal inhibition by the agonist and EC_{50} is the concentration of agonist producing half-maximal inhibition, or $(R_i + R_0)/2$. This equation was derived as follows: the maximal effect of the agonist is the difference between the maximal rate of activity and the maximally inhibited rate, $R_0 - R_i$, and the effect of a particular concentration of agonist is $R_0 - R$. The fractional response is assumed to be proportional to receptor occupancy, yielding:

$$\frac{(R_0 - R)}{(R_0 - R_i)} = \frac{[A]}{([A] + EC_{50})} \qquad (2)$$

solving for R yields equation 1. Inclusion of a slope factor, n, as an exponent of the [A] and EC_{50} parameters yields:

$$R = R_i - (R_i - R_0) / [([A] / EC_{50})^n + 1] \qquad (3)$$

b) Stimulation concentration-response curves: For stimulation, a similar

equation was derived from the folowing:

$$\frac{(R - R_b)}{(R_s - R_b)} = \frac{A}{A + EC_{50}} \qquad (4)$$

where R_b is basal adenylyl cyclase activity, R_s is maximally stimulated rate, and R is the stimulated rate at each concentration of agonist, [A]. Rearrangement of (4) yields:

$$R = R_s + (R_b - R_d) / [([A] / EC_{50}) + 1] \qquad (5)$$

In the case of two noninteracting populations of receptors, the stimulation can be described by the sum of two logistic equations with a constant indicating the proportion of the response attributable to each population.

$$\frac{(R_s - R)}{(R_s - R_b)} = \frac{f\,[A]}{[A] + C_H} + \frac{(1-f)\,[A]}{[A] + C_L} \qquad (6)$$

where f represents the proportion of the response contributed by either of the two receptors, and C_H and C_L represent the EC_{50} values of the two receptors. Let $\alpha = (A/C_H)$ and $\beta = (A/ C_L)$.

$$R - R_b = \frac{f(R_s - R_b)\,\alpha}{\alpha) + \alpha)} + \frac{(1-f)(R_s - R_b)\,\beta}{(1 + \beta)} \qquad (7)$$

The first term in equation (7) can be expanded to:

$$\frac{(fR_s\alpha - fR_b\alpha)}{(1+\alpha)} = \frac{(fR_s\alpha - fR_b\alpha + fR_b - fR_b + fR_s - fR_s)}{(1+\alpha)} \qquad (8)$$

Equation (8) is equal to:

$$fR_s - fR_b + \frac{fR_s - fR_b}{a) + \alpha)} \qquad (9)$$

Similar expansion of the second term of (6), combination of both terms and solving for R yields the following expression:

$$R = R_s + \frac{f(R_b - R_s)}{1 + ([A]/C_H)} + \frac{f\,(R_b - R_s) + R_b - R_s}{1 + ([A]/C_L)} \qquad (10)$$

Equation (10) was fit to the data in cases where the response resulted from the stimulation of two receptor populations.

c) Time course equations: The decline in the stimulated rate of adenylyl cyclase activity over time can be described as the result of two independent processes: a decline in the system (reflected in the decline in the basal adenylyl cycalse activity) and a 5-HT stimulated decay. The system decay is described adequately by simple first order decay:

$$R_{bt} = R_{bo} \exp(-k_{sys}t) \qquad (11)$$

where k_{sys} a first order decay constant for the system, t is time, R_{bt} is the basal rate at any time t and R_{bo} is the basal rate at time 0. The receptor-

stimulated rate at time t, R_{st}, can be represented as a constant, A, times the basal rate:

$$R_{st} = A \times R_{bt} = A \times R_{bo} (-k_{sys}t) \qquad (12)$$

Equation (12) is used to describe the stimulated rate of adenylyl cycalse activity by an agonist over time with no additional decay mechanism occurring, as with histamine stimulated adenylyl cyclase activity. However, in some cases (as with 5-HT), there is an additional route of decay stimulated by the drug itself.

$$R_s \xrightarrow{k_{ds}} R_d \qquad (13)$$

where R_s the rate produced by receptor stimulation, R_d represents the rate of the "desensitized" receptor, and k_{ds} is the rate constant. The rate of adenylyl cyclase activity is proportional to the number of receptors in each form, and the total rate is the sum of both sensitized and desensitized rates.

$$R_s + R_d = R_o \qquad (14)$$

where R_o is the observed rate of adenylyl cyclase activity at t = 0. From (13) and (14), R_d can be calculated to be:

$$R_d = R_o(1 - \exp(-k_{ds}t)) \qquad (15)$$

If A represents the fold-stimulation of basal activity by the sensitized

receptors and B the fold-stimulation by sensitized ones, the rate of adenylyl cyclase activity at any time t can be expressed as the sum of :

$$Y_t = A \times R_{bo} \exp(-k_{ds} x t) + B \times R_{bo}(1 - \exp(-k_{ds} t)) \qquad (16)$$

If B/A is replaced by F_d, which represents the fractional activation by the desensitized form of the receptor, then rearrangement of (16) yields:

$$Y_t = A \times R_{bo} [(1-F_d) \exp(-k_{ds} t) + F_d] \qquad (17)$$

Combining (17) with (12), which express the decay in basal activity, yields:

$$Y_t = A \times R_{bo} \exp(-k_{sys} t) \times ((1-F_d)\exp(-k_{ds} t) + F_d) \qquad (18)$$

Equations (18) and (12) were used to describe the time course data in figure 26.

d) Antagonism data: The K_b values of abntagonists were estimated by the method of Arunlakshana and Schild (1959) when different concentrations of antagonists were used, or by the method of Gaddum (1937) when single doses of antagonists were employed.

$$\log(dr-1) = n \cdot \log[B] - \log K_b \qquad (19)$$

$$p = \frac{1}{1 + (K_a/[A])(1 + [B]/K_b)} \quad (20)$$

where dr is the dose ratio, or the ratio of equiactive concentrations of an agonist, A, [B] is the concentration of antagonist producing the dose ratio, K_a and K_b are the dissociation constants of the agonist and antagonist, n is the slope of the Schild plot and p is the fractional receptor occupancy by the agonist.

All curve-fitting was done with unweighted, nonlinear regression analysis using FITFUN, a computer modeling program available on the PROPHET Computer System.

III. RESULTS

III. 1 Effects of forskolin and 5-HT on adenylyl cyclase activity

Forskolin, with an EC_{50} value of approximately 0.4 µM, stimulated adenylyl cyclase (AC) activity in guinea pig hippocampal membranes 4 to 5-fold (fig. 2). At low concentrations of forskolin (< 0.1 µM), 5-HT (10 µM) stimulated AC activity by 85%. Conversely, at forskolin concentrations ≥ 10 µM, 5-HT inhibited AC activity by 30%. The maximal percentage of inhibition by 5-HT was approximately 30% in the presence of either 10 µM or 100 µM forskolin. Based on these experiments, a concentration of forskolin of 10µM was chosen to study the inhibition of adenylyl cyclase activity. Stimulation of basal AC activity was measured in the absence of forskolin.

The increase in AC activity in the presence of 10 µM forskolin was linear with respect to time and protein concentration (fig.3 A,B). The linearity of the assay depended on the concentration of the enzyme creatine phosphokinase (CPK) (fig. 3 C). On the basis of these experiments, an assay time of 5 minutes and 15 seconds, a CPK concentration of 10 µg/ assay and a membrane concentration of 20 to 60 mg of protein per assay (approximately 150 µg per ml) were chosen and all other experiments measuring inhibition were done under these conditions.

Inhibition of forskolin-stimulated adenylyl cyclase activity in guinea pig hippocampal membranes by 5-HT is shown in fig. 4A. 5-HT elicited a decrease in AC activity of approximately 50 pmols cAMP/mg protein/min.,

and the standard error of the mean (SEM) of the points averaged 1.5 pmols cAMP/ mg protein /min. Dividing the decrease by the SEM yielded a high signal to noise ratio of approximately 30, making this preparation suitable for accurately measuring concentration-response curve parameters (see section I.3). AC activity was inhibited by 30% over a concentration range of 5-HT of 10^{-9} to 10^{-7} M. The effect of 5-HT was saturable and there was no evidence suggesting that multiple receptors were involved in generating the response. The three parameter logistic equation (equation 1), an equation that is derived from the interaction of a drug with a single, homogeneous population of non-interacting receptors (see methods), fit the data with a high correlation of variation (R^2 value of 0.96) (4B, solid line). Introduction of a fourth parameter into the equation, a slope factor, as an exponent of the $[A]/EC_{50}$ ratio (equation 3), reduced the residual error of the fit (4B, dashed line). The correlation of variation of the the 4 parameter equation was nearly identical to the 3 parameter equation ($R^2 = 0.97$). The F test of the improvement of the fit by the introduction of the slope factor indicated that the fit of the data was significantly improved by the introduction of the slope factor at a probabilty level of $0.05 < p < 0.02$. However, examination of the residual error pooled from seven 5-HT concentration-response curves fitted with and without a slope factor (De Lean et al., 1978) resulted in a nonsignificant improvement of the fit by introduction of a slope factor ($p > 0.05$). Therefore, based on these results, all other experiments were fitted with equation 1 and no obvious deviations from the theoretical curves was observed, except with lisuride and guinea pig hippocampal membranes (see below). The geometric mean of the EC_{50} values for 5-HT in guinea pig hippocampal membranes was 53 nM (range: 8.6 to 231 nM) and the mean percentage of inhibition was 31% (range: 24 to 35%).

III.2 Characterization of the receptor mediating inhibition of AC activity

The 5-HT_{1A}-selective ligands PAT and buspirone and the 5-HT_1 selective ligand RU 24969 were potent in inhibiting forskolin-stimulated AC activity in guinea pig hippocampal membanes (fig. 5). The tryptamine derivatives 5-CT and 5-methoxytryptamine (fig. 6), the piperazines PAPP and TFMPP (fig. 7), and the ergot derivatives LSD, methysergide and metergoline (fig. 8) were all potent agonists in inhibiting AC activity with EC_{50} values < 300 nM (table 1). The agonists tested on rat hippocampal membranes were similarly potent in inhibiting forskolin-stimulated AC activity (table 1, fig. 10). [Note: some of the experiments done to determine the parameter values in rat membranes were done by Barbara Royal and not by me, but all data analyses were performed by me].

Spiperone (fig. 10A) and methiothepin (fig. 10B) were potent competitive antagonists of the 5-CT-mediated inhibition of AC activity. 5-CT concentration-response curves in the presence of either antagonist were shifted to the right with no decrease in the percentage of inhibition, although forskolin-stimulated activity increased in the presence of each antagonist. Schild regression analysis (fig. 11) of the antagonism resulted in the following estimates of K_b values ± SD: 13 ± 1 nM (methiothepin) and 26 ± 1 nM (spiperone). Standard deviations were calculated from the standard deviation of the regression as described by De Lean et al. (1981). Pindolol, both the racemic mixture and the (-)isomer, were tested and found to be partial agonists in guinea pig and rat hippocampal membranes. The

activity of pindolol was small, however, (i.a. < 0.1), therefore the drug was tested as an antagonist. The K_b of (-) pindolol was 40 ± 4 nM in rat hippocampal membranes (fig. 12). Ketanserin (fig. 13) and ICS 205-930 (not shown), at concentrations of 1 μM, were ineffective in antagonizing the response to 5-CT.

Inhibition of adenylyl cyclase by 5-HT_{1A} receptors was measured in rat cerebral cortical membranes. The inhibition of forskolin-stimulated AC activity by the 5-HT_1 selective ligand 5-CT and 5-HT_{1A} selective ligand buspirone is shown in fig. 14A. The mean EC_{50} value of 5-CT was 11 ± 2 nM and the mean maximal percentage of inhibition was 17 ± 3% (n = 4). Buspirone was a partial agonist in this tissue, with a mean percentage of inhibition that was < 10% of total forskolin-stimulated activity and an EC_{50} of approximately 0.5 μM. Because of the low percentage of inhibition by buspirone, it was also tested as an antagonist in rat cerebral cortical membranes. Buspirone, at a concentration of 10 μM, shifted the concentration-response curve to 5-CT to the right by 10-fold, indicating that its K_b value was approximately 1 μM (fig. 14B). Both PAT and 5-HT also potently inhibited forskolin-stimulated AC activity in rat cerebral cortical membranes, with EC_{50} values < 100 nM (data not shown).

In rat hippocampal membranes, lisuride was the most potent agonist tested (table 1 and fig. 10). However, in guinea pig hippocampal membranes, lisuride inhibited forskolin-stimulated AC activity at concentrations as low as 10 pM, but the data could not be fit with standard concentration-response curves (fig. 15A). In the experiment shown in fig. 15A, the concentration-response data could be fit only with the introduction of a fourth parameter into the equation, a slope factor (equation 3). The computer-derived values for n, the slope factor, was 0.44, the EC_{50} was 80 pM and the maximal

percentage of inhibition was 43%. This effect of lisuride was reproducible in six experiments and was not affected by increasing the time of incubation of the assay (data not shown). However, by diluting the receptors with spiperone, lisuride did inhibit activity in a conventional way (i.e. the data were fit with equation 1 with a high correlation of variation) (fig. 15B). The EC_{50} of lisuride in the presence of 1 µM was used to estimate the EC_{50} of lisuride if the data had conformed to conventional concentration-response curve analysis. This value was approximately 10 nM.

III.3 Stimulation of adenylyl cyclase activity by 5-HT

5-HT and PAT stimulated basal AC activity under both the total homogenate and crude membrane (fig. 16) assay conditions. Using the total homogenate conditions, stimulation was nearly equally mediated by two separate receptors (Shenker et al., 1985). Using the crude membrane preparation, the PAT- stimulated activity was reduced and sometimes abolished, but 5-HT-stimulated activity was still apparent. TFMPP, a 5-HT_{1A} agonist, did not stimulate basal AC activity at concentrations up to 100 µM. Increasing the concentration of membrane protein in the crude membrane assay resulted in a restoration of 5-CT-stimulated activity in some experiments (fig. 17). This effect of reduced membrane concentration did not occur routinely, as, in other experiments, 5-CT stimulated AC activity even at low concen-trations of membrane protein (data not shown). Histamine stimulated AC activity both in the presence and absence of forskolin (fig. 18).

Using the crude membrane preparation with low membrane

concentrations, 5-HT stimulated basal AC activity approximately 100% with an EC_{50} value of 200 ± 40 nM (mean ± SEM of three experiments). Spiperone, fluphenazine and mianserin competitvely antagonized the response to 5-HT (fig. 19). No slope factor was included in the analysis of the concentration-response data. The percentage of stimulation of basal adenylyl cyclase activity by 5-HT was nearly the same in the absence and presence of the antagonists in all experiments. The K_b values of the antagonists were estimated from the shift of the EC_{50} value of 5-HT in the presence of a 10 µM concentration of each antagonist. The K_b values ± SEM were: 1.78 ± 0.09 µM (mianserin), 400 ± 45 nM (fluphenazine) and 168 ± 8 nM (spiperone) (results are the geometric means of three experiments). The contribution of the $5\text{-}HT_{1A}$ receptor to the stimulation was small (< 20%) as monitored by measuring the stimulation produced by selective $5\text{-}HT_{1A}$ agonists, such as PAT and PAPP. Although small, the contribution of this receptor was always present and therefore, probably influenced the K_b values obtained in these experiments.

III.4 Effects of 5-HT on stimulation or inhibition of AC by GTP and Mg^{++}

GTP, in the absence of 5-HT, did not inhibit forskolin-stimulated AC activity at concentrations up to 100µM (fig. 20A). In the presence of 10 µM 5-HT, GTP inhibited AC activity in two experiments by 32% and 33% and with EC_{50} values of 1.2 and 1.7 µM. In contrast, Gpp(NH)p inhibited AC activity by approximately 50% in the presence or absence of 5-HT (fig. 20B). The EC_{50} values of Gpp(NH)p were 1.1 µM and 0.51 µM in the absence of 5-HT

and 0.55 µM and 0.33µM in the presence of 5-HT. 5-HT did not inhibit AC activity in the absence of exogenous GTP (fig. 20A). In contrast, GTP stimulated basal activity in the absence of 5-HT (fig. 21A) and 5-HT stimulated basal activity in the presence of Gpp(NH)p (fig. 21B). The EC_{50} value of GTP was approximately 1 µM in three experiments of this type, and GTP stimulated activity by 2 to 3-fold in all experiments. In the presence of 5-HT, GTP stimulated basal activity by 4 to 5-fold with nearly the same EC_{50} value.

5-HT inhibited forskolin-stimulated AC activity by 27% in the presence of small (approximately 1 µM) concentrations of Mg^{++} (fig. 22). The free concentration of Mg^{++} was estimated by subtracting the concentration of ATP and EDTA from the total concentration of Mg^{++} added. At the highest concentration of Mg^{++} tested (10 mM), 5-HT inhibited AC activity by 12%. Mg^{++} increased forskolin-stimulated activity by 10-fold in the absence or presence of 5-HT. In the absence of forskolin, Mg^{++} increased basal AC activity by 20-fold (fig. 23). The EC_{50} of Mg^{++} in stimulating basal activity was reduced from about 6 to 3 mM by 10 µM 5-HT. 5-HT did not alter the EC_{50} of Mg^{++} in stimulating AC activity in the presence of forskolin (4.1 µM in the absence and presence of forskolin; fig. 22).

III. 5 Effects of reserpine on the stimulation and inhibition by 5-HT of AC activity

Reserpine, at doses of 5 mg/kg body weight, was administered i.p. to guinea pigs. The EC_{50} of 5-HT in inhibiting AC activity in membranes from reserpinized animals was significantly reduced compared to control animals

that received saline injections (fig. 24). The reduction in EC_{50} values in these paired experiments were: from 46 to 16 nM, from 31 to 18 nM and from 50 to 15 nM. The maximal percentage of inhibition for the reserpined animals averaged 31%, the same as in untreated animals (table 1). In three experiments with buspirone, reserpine reduced the EC_{50} in two animals compared to the control (106 to 28 nM and 410 to 64 nM). In the third pair, the EC_{50} was not reduced (from 42 to 62 nM) but the percentage of inhibition was significantly increased from 23% to 30% (data not shown).

The maximal stimulation by 5-HT of AC activity in hippocampal homogenates from reserpine-treated guinea pigs is increased significantly with respect to homogenates from paired control animals, although reserpine treatment does not affect histamine stimulation of AC activity (Shenker et al., 1983). In the experiment shown in fig. 25, reserpine doubled the maximal percentage of stimulation by 5-HT of AC activity in guinea pig hippocampal total homogenates. Resolution of the concentration-response data into the separate contributions of two receptors (5-HT_{1A} and R_L) indicated that both receptors mediating stimulation in the total homogenate preparation were equally affected by reserpine (in three experiments, the average stimulation by 5-HT_{1A} receptors was approximately 50%). Administration of the 5-HT precursor 5-HTP reduced the stimulation by both receptors by approximately 50% in seven experiments (fig. 25). The average stimulation by histamine was the same in homogenates from control (175%) and 5-HTP-treated (176%) guinea pigs (data not shown).

III.6 Decrease in 5-HT-stimulated AC activity with time

The fold-stimulation by 5-HT of adenylyl cyclase activity over basal activity in homogenates from guinea pig hippocampi decreased with time to a rate that was approximately 50% of the maximal percentage of increase by 5-HT at time zero (fig. 26). Basal and histamine-stimulated activity also decreased with time, but the histamine-stimulated rate was always a constant-fold stimulation of the basal rate (in fig. 26, the fold stimulation by histamine was constant throughout the 60 minute time of the experiment). Addition of 5-HT during the incubation did not restore the responsiveness of the homogenate to 5-HT. Incubating the membranes without 5-HT and adding 5-HT at each time period resulted in a constant fold-stimulation of basal activity. Rate constants for both the decay in basal activity and for the 5-HT inhibition of 5-HT stimulated activity were calculated from six experiments. The rates ± SD (n = 6) were: 0.018 ± 0.006 (basal), and 0.36 ± 0.14 (5-HT).

The decrease in 5-HT stimulated activity was dependent upon the concentration of 5-HT present during the preincubation. Total homogenates were incubated with various concentrations of 5-HT for 30 min. and then exposed to a concentration of 5-HT (10 µM) that elicits a maximal response. The decrease in 5-HT stimulated activity in response to the concentration of 5-HT during pre-incubation is shown in fig. 27. The decay was dependent on the presence of GTP and Mg^{++}. In the absence of GTP and Mg^{++}, 5-HT stimulated activity was equal whether 5-HT was present during the incubation or not (fig. 28A). In membranes from 5-HTP-treated guinea pigs, incubation of the membranes with 5-HT resulted in a further reduction in the maximal stimulation by 5-HT, from an average of 50% to 25% (fig. 28B).

III.7 Effects of pertussis toxin on the inhibition of AC activity by 5-HT

Incubation of guinea pig hippocampal membranes with 10 µg of pertussis toxin (PT) reduced the maximal percentage of inhibition by 5-HT of forskolin-stimulated activity from 22% to 16% in membranes from a single animal (fig. 29). Increasing the time of incubation resulted in a loss in activity of both the PT-treated and control membranes (conditions for the incubation of membranes with PT were chosen from Okajima et al., 1985). PT, admistered in vivo to rats stereotaxically, reduced both the inhibition of forskolin-stimulated AC activity and the decrease in population spike amplitude produced by 5-HT in slices of rat hippocampus. [Note that the electrophysiological measurements were made by William Clarke and not by me, they are included in this section for ease of comparison only. All adenylyl cyclase experiments were done by me.] In a rat pretreated with 0.5 µg PT, 5-HT inhibited AC activity 32% and decreased the PSA by 50%, However, 1.5 µg of PT reduced the maximal percentage of inhibition of AC activity by 5-HT to 16% and the decrease in PSA produced by 10µM 5-HT to 9% (fig. 30). At higher doses of PT (3 µg), the 5-HT-mediated inhibition of AC activity was completely abolished (fig. 31). The decrease in maximal percentage of inhibition by 5-HT was significantly correlated with the dose of PT administered (fig. 32). Among all animals tested, the decrease in PSA by 10 µM 5-HT and the maximal percentage of inhibition of AC activity by 5-HT were significantly correlated (fig. 33). For correlations of the percentages, the data were first transformed by the arcsine transformation (Zar, 1984), because data expressed as percentages are not normally distributed and correlation tests are only applicable with normally

distributed data.

IV. DISCUSSION

IV.1 Effects of 5-HT and forskolin on adenylyl cyclase activity

In guinea pig hippocampal membranes, the net effect of 5-HT on adenylyl cyclase (AC) activity depends upon the preexisting level of stimulation of AC. Increasing concentrations of forskolin reversed the net effect of 5-HT on AC activity from stimulation to inhibition (fig. 2). Forskolin, in addition to stimulating the catalytic unit directly, may also enhance stimulation by G proteins (G_s) (Bender et al., 1985). The EC_{50} value of forskolin (0.3 µM) (fig. 2) in stimulating AC activity was smaller than forskolin's EC_{50} value in stimulating the purified catalytic unit of AC (approximately 20 µM) (Bender et al., 1985). The low EC_{50} value may reflect this secondary action of forskolin in enhancing stimulation by G_s.

The reversal of the net effect of 5-HT was not a nonspecific effect of forskolin stimulation because histamine stimulated AC activity both in the absence and presence of forskolin (fig. 18). The opposing effects of 5-HT on AC activity suggests the presence of multiple 5-HT receptors in guinea pig hippocampal membranes, both positively and negatively coupled to AC. The overall effect of 5-HT was to dampen the amplification of AC activity by forskolin. Forskolin stimulated basal activity 5-fold, but only doubled activity in the presence of 5-HT (fig. 2).

IV.2 Classification of 5-HT receptors negatively coupled to AC

Classification of the receptor mediating inhibition of AC activity in guinea pig hippocampal membranes is relatively straightforward because of the high signal to noise ratio obtained with this preparation (section III.1) and the simple nature of the concentration-response curves describing the data. 5-HT concentration-response data were adequately fit with equations derived from the law of mass action (fig. 4B ; equation 1), without introduction of a "slope factor" as an exponent of the $[A]/EC_{50}$ ratio. The reasons for fitting the concentration-response data without a slope factor were: visual inspection of the data indicated that the points are essentially evenly distributed around curves drawn with and without slope factors (fig. 4B); correlation of variation (R^2) values for the goodness of fit of the equations for the data were essentially identical with and without inclusion of a slope factor; and an F test of seven pooled experiments demonstrated that inclusion of a slope factor did not significantly improve the fit of the equation to the data (section III.1). Antagonists competitively shifted the concentration-response curves with no decrease in the percentage of inhibition by agonists (fig. 10, 11), further supporting the contention that the respone was mediated by a single, homogeneous population of receptors.

Concentration-response data (table 1) satisfy the criteria established by Bradley et al. (1986) in order for a response to be classified as mediated by 5-HT_1 receptors. 5-CT and methysergide were potent agonists (fig. 6,8) and the response was potently antagonized by methiothepin (fig. 11), but not by ketanserin (fig. 13) or ICS 205-930 (data not shown). In addition, the data indicate that the response is mediated by a receptor homologous with the 5-HT_{1A} binding site, because 5-HT_{1A} selective ligands such as PAT (Middlemiss and Fozard, 1983), buspirone (Glaser and Traber, 1983), and

PAPP (Ransom et al., 1985) all inhibited forskolin-stimulated AC activity with EC_{50} values < 200 nM (fig. 5,7 and table 1). EC_{50} values were highly correlated with the reported affinities of these ligands for 5-HT_{1A} binding sites (r = 0.994, Schlegel and Peroutka, 1986; r = 0.92, Engel et al., 1986; r = 0.95, Ransom et al., 1986a,b). Spiperone, a ligand used to define 5-HT_{1A} binding sites (Pedigo et al., 1981), was a potent, competitive antagonist of the response (K_b = 26 nM) (fig. 10,11).

Results obtained with rat hippocampal membranes were similar to those obtained with guinea pig tissue. (fig. 9; table 1). All EC_{50} values obtained with rat hippocampal membranes were higher than those in guinea pigs, and the intrinsic activity of the partial agonist buspirone was reduced in half. . The response of rat membranes to 5-HT was competitively antagonized by (-)pindolol (fig. 12), a drug that blocks behavioral effects of 5-HT_{1A} receptor stimulation (Tricklebank et al., 1985), supporting the classification of the inhibitory receptor as 5-HT_{1A}. Rat cerebral cortical membranes also contain relatively high densities of 5-HT_{1A} binding sites (Pedigo et al., 1981). In rat cerebral cortical membranes, 5-CT (fig. 14), 5-HT, PAT and buspirone inhibited forskolin-stimulated activity, demonstrating that 5-HT_{1A} receptors are negatively coupled to AC in rat cerebral cortical as well as hippocampal membranes.

Rat hippocampal membranes contain a lower density of [^{3}H]5-HT binding sites than guinea pig membranes, and most of these sites probably represent 5-HT_{1A} binding sites (Schnellmann et al., 1984). According to pharmacological theory, reducing the number of receptors in a tissue produces, at first, a shift to the right in the concentration-response curve of full agonists and a decrease in the intrinsic activity of partial agonists (Stephenson, 1956). Therefore, the difference in EC_{50} values and intrinsic

activity between guinea pig and rat membranes may reflect a difference in receptor number. Assuming that the function relating stimulus to response is the same in both guinea pig and rat hippocampus, the Stephenson model could be used to calculate the K_d of an agonist if the relative number of receptors were known in the two tissues. The reduction in receptor number in rat compared to guinea pig hippocampal membranes can be estimated by the reduction in the intrinsic activity of the partial agonist buspirone (42%; table 1). This decrease is close to the difference in number of 5-HT_1 binding sites measured in the two tissues, approximately 25% (Schnellmann et al., 1984). Rough approximations of the K_d of 5-HT yields an approximate value of 300 nM, much higher than reported affinities of 5-HT for 5-HT_{1A} sites measured in binding experiments (13 nM, Sills et al., 1984). A possible reason for this discrepany is that the affinity measured in binding studies (even in the presence of GTP) represents both coupled and uncoupled receptor populations and, in functional studies, only receptors that cycle through the G protein systems are measured. The off-rate of the drug from the coupled receptor is presumably controlled by the GTPase step in AC activation, not by the dissociation rate of the drug from the receptor as in binding studies. Therefore, a value of 300 nM is more likely to represent the affinity of 5-HT for functional 5-HT_{1A} receptors. In light of this, it is not surprising that physiological effects of 5-HT_{1A} receptor-stimulation occur at concentrations of 5-HT above 300 nM (Andrade et al., 1986; Beck et al., 1985). Leysen (1984) claimed that the nanomolar affinity of 5-HT for 5-HT_1 binding sites argued against these binding sites being physiolog-ically relevant since these sites would be saturated at all times by endogenous 5-HT. Therefore, the demonstration that the affinity is of the order of 300 nM is important in establishing the physiological relevance of the 5-HT_{1A}

receptor.

The results from this study unambiguously identify agonists of the $5\text{-}HT_{1A}$ receptor and thereby permit reevaluation of conclusions that have been made in the past concerning the effects of these agonists on other systems. Both metergoline and methysergide (fig. 8), for example, are agonists at the $5\text{-}HT_{1A}$ receptor, but are often generally assumed to be 5-HT receptor antagonists. Sprouse and Aghajanian (1986) reported that metergoline did not block the inhibition of raphe firing elicited by 5-HT, but depressed firing by itself, and they attributed this phenomenon to a nonspecific effect rather than to agonism by metergoline. Similarly, metergoline and methysergide exhibit "paradoxical 5-HT agonist properties" in that both decrease turnover of 5-HT in rat brain (Bourgoin et al., 1978), a characteristic of agonists, but not antagonists such as methiothepin (Bourgoin et al., 1977). Lisuride and LSD (Bourgoin et al., 1978), PAT (Arvidsson et al., 1981), buspirone (Hjorth and Carllson, 1982), TFMPP and RU 24969 (Fuller, 1984), all decrease 5-HT turnover and are agonists at $5\text{-}HT_{1A}$ receptors. An oddity concerning drug development for 5-HT receptors is that, while many antagonists have high affinity for $5\text{-}HT_2$ receptors, most high-affinity ligands developed for $5\text{-}HT_1$ sites are agonists (Hamon et al., 1984; e.g.). Since measurements of 5-HT turnover in vivo is commonly used to screen drugs for potential interactions with 5-HT receptors in brain, however, <u>only</u> drugs which are agonists can be identified. Potent $5\text{-}HT_{1A}$ antagonists that have been developed, until recently, were previously identified as antagonists of peripheral smooth muscle receptors other than $5\text{-}HT_1$ (e.g.,methiothepin, spiperone and pindolol). By measuring inhibition of AC activity, identification of new, selective $5\text{-}HT_{1A}$ antagonists should be possible.

IV. 3 The "lisuride anomaly"

Lisuride exhibited anomalous behavior in guinea pig hippocampal membranes (fig. 15A). Concentration-response data obtained with lisuride could not be analyzed with equation 1, whereas data obtained with all other agonists were fit with equation 1 with a high correlation of variation (R^2). Lisuride did exhibit "normal" behavior in rat hippocampal membranes, where it was the most potent agonist tested (fig. 9, table 1). Therefore, the lisuride anomaly in guinea pig membranes may result from its high affinity for 5-HT_{1A} receptors. In generating responses in AC systems, two equilibria are important: the equilibrium between drug and receptor, and the equilibrium between the drug-receptor complex and the effector, or G protein (section I.7). The off-rate of the drug-receptor complex from G is presumably governed by the GTPase activity of the G protein: after hydrolysis of GTP, the drug-receptor complex dissociates from G and the drug dissociates from R. Perhaps the high affinity of lisuride disrupts that equilibrium, with lisuride staying bound to the receptor even after dissociation of the drug-receptor complex from G. Therefore, lisuride may act as an irreversible agonist in this system, not dissociating from the receptor at the same point in the cycle as other agonists do. This explanation is consistent with the observations that lisuride is remarkably potent (it inhibits AC activity at concentrations as low as 10 pM), and achieves the highest percentage of inhibition of any agonist, 43% in the experiment shown in fig. 9. This explanation may account for the shallow concentration-response curves obtained with lisuride (n = 0.44 in fig. 15A),

since a drug-receptor complex that remained active after dissociating from an active G protein could exhibit negative cooperativity in eliciting its response if the number of inactive G proteins is constantly reduced during the time the drug is bound to the receptor. Reducing the apparent affinity of lisuride by diluting the receptors with spiperone eliminates lisuride's anomalous behavior (fig. 15B), supporting the idea that this behavior results from the high affinity of lisuride, and not from any changes induced by lisuride on the G proteins or from any nonspecific effect of lisuride on the AC system.

IV. 4 Classification of 5-HT receptors positively coupled to AC

5-HT stimulates basal AC activity in guinea pig homogenates by two distinct receptors (Shenker et al., 1985). The receptor with higher affinity for 5-HT appears to be homologous with the 5-HT_{1A} binding site; whereas the other receptor does not resemble a known binding site and is designated R_L. Therefore, 5-HT_{1A} receptors both stimulate and inhibit AC activity in the same tissue. Using the crude membrane preparation as compared to the total homogenate conditions used by Shenker et al., stimulation of AC activity by PAT was reduced (fig. 16), and in some experiments, abolished. The crude membrane preparation routinely contains a lower concentration of membrane protein than the total homogenate preparation used by Shenker et al.. This difference may result in the loss of 5-HT_{1A} receptor-stimulated AC activity, because increasing the membrane concentration restored 5-CT-stimulated activity in some experiments (fig. 17). Histamine and R_L-stimulated activity was largely unaffected by changes in membrane

concentration. Although in some experiments with low membrane concentration 5-HT_{1A} stimulation was abolished, this was not true in all cases. The stimulation by 5-HT_{1A} receptors was variable with either of the two assay conditions used. In contrast, inhibition by 5-HT_{1A} agonists was always greater than 24% of forskolin-stimulated activity under either set of assay conditions. Stimulation of AC activity by 5-HT_{1A} receptors in rat hippocampal membranes was reported by Markstein et al. (1986). However, the SNR of their results was low (approximately 6, see section I.3 for an explanation of SNR) and their results correlate poorly (r= 0.55) with the results in table 1. Obvious differences between the results reported by Markstein et al. and those in table 1 include: the high EC_{50} values of RU 24969 (> 1 µM), LSD and lisuride (300 to 400 nM), and the low K_b values of methiothepin and spiperone, 2 to 3 nM, which were 10-fold lower than results obtained with guinea pig hippocampal membranes .

In intact tissues, evidence suggests that 5-HT_{1A} receptors are negatively coupled to AC. Weiss et al. (1986) measured an inhibition by PAT of vasoactive intestinal peptide-stimulated cAMP formation in intact murine striatal and cortical cells. They reported that PAT did not stimulate cAMP formation in those cells, although 5-HT, through a different receptor, did stimulate cAMP formation. 5-HT_{1A} receptor stimulation produces electro-physiological effects opposite to that produced by agents that mimic the actions of cAMP in the rat hippocampal slice (discussd below). Lesioning of serotonergic axons in rat forebrain increases isoproterenol-stimulated cAMP formation in hippocampal minces (Stockmeier et al., 1985), an effect that could result from removal of 5-HT. The physiological responses to 5-HT_{1A} receptor stimulation apparently depend upon coupling of the receptor to a pertussis toxin-sensitive substrate (Clarke et al., 1986; Andrade et al.,

1986; discussed below). 5-HT_{1A} receptor-mediated stimulation of cAMP formation has not been shown in an intact system, although 5-HT_1 receptors may stimulate cAMP formation in porcine vena cava (Trevethick et al., 1986). In guinea pig hippocampal membranes, as discussed above, the magnitude of the 5-HT_{1A} mediated stimulation of basal activity is highly variable compared to the inhibitory response, and is sometimes not present at all. In rat cerebral cortical membranes, a tissue with a high concentration of 5-HT_{1A} binding sites, 5-HT_{1A} receptor-mediated inhibition of AC activity was measured (fig. 14) but no stimulation by 5-HT (in the absence of forskolin) could be detected at concentrations of 5-HT up to 20 µM (data not shown). Based on the variable stimulatory 5-HT_{1A} response in hippocampal membranes, on the absence of a stimulatory response in cerebral cortical membranes, and on the results from intact cells and slices, it is likely that 5-HT_{1A} receptors are preferentially <u>negatively</u> coupled to adenylyl cyclase. Until 5-HT_{1A} receptor-stimulated cAMP formation can be unambiguously demonstrated in an intact cell or slice, such a role for this receptor must be considered speculative. Asano et al. (1984) demonstrated that ß-adrenergic receptors could couple to G_i in reconstituted vesicles, although these receptors do possess a high degree of selectivity for G_s. Perhaps 5-HT_{1A} receptors couple to stimulatory regulatory proteins when the concentration of membrane protein (and, therefore, the concentration of receptors and regulatory proteins) is high enough to permit this cross-activation phenomenon to occur.

Stimulation of AC activity by 5-HT was measured under the crude homogenate conditions, limiting 5-HT_{1A} stimulation, in order to characterize the lower affinity 5-HT receptor (R_L) positively coupled to AC. Two pharmacologically distinct 5-HT receptors positively coupled to AC in

mammalian brain have been characterized: the 5-HT receptor that stimulates AC activity in membranes from infant rat colliculi and the receptor found on NCB-20 cells (derived from mouse neuroblastoma and hamster brain cells). The most potent antagonist reported for the the collicular receptor is spiperone (Nelson et al., 1980 a,b), and the most potent antagonists reported for the NCB-20 cells are mianserin and fluphenazine (MacDermot et al., 1979). These receptors appear to be distinct because spiperone, at concentrations up to 100 µM, does not antagonize the response of the NCB-20 receptor in intact cells (Berry-Kravis and Dawson, 1983). In order to compare the R_L receptor with these two receptors, the effects of the antagonists spiperone, fluphenazine and mianserin were measured (fig. 19). The K_b values of mianserin (1.78 µM), fluphenazine (400 nM), and spiperone (168 nM) were in good agreement with the results obtained by Nelson et al. in infant rat collicular membranes (1.3 µM, 550 nM and 180 nM, respectively). The K_i values for the infant rat collicular receptor were calculated by converting IC_{50} values by the Cheng-Prusoff equation (1973), using 0.5 µM as the EC_{50} of 5-HT. An additional similarity between the collicular receptor and R_L is that TFMPP does not stimulate AC activity at R_L (not shown), as it does not at the collicular receptor (Nelson et al., 1980a). The pharmacology of the R_L receptor does not resemble the 5-HT_1 class, since 5-CT appears inactive, or the 5-HT_2 class, because of the low affinity of spiperone. Shenker et al. (1985) proposed that the R_L receptor resembles the infant rat collicular receptor based on the EC_{50} value of 5-HT. The results from this study support that classification. 5-HT-stimulated AC activity has been reported in many intact cell and broken cell membrane preparations, with EC_{50} values in the 100 nM to 1 µM range, indicating that 5-HT receptors positively coupled to

AC are widely distributed throughout the CNS and periphery (reviewed in section I. 3).

IV. 5 GTP and Mg^{++}-mediated effects on AC activity

In addition to characterizing changes in AC activity with agonists and antagonists acting at the receptor, changes in AC activity can be, to a limited extent, characterized with respect to GTP and Mg^{++}. Under the assay conditions used in the experiments reported here, there was a characteristic difference between inhibition and stimulation of AC activity with respect to GTP. GTP, by itself, did not appreciably inhibit AC in the absence of 5-HT (fig. 20A), although GTP did stimulate basal AC ativity in the absence of 5-HT (fig. 21A). The small decrease in AC activity observed at high concentrations of GTP in fig. 20A may result from chelation of Mg^{++}, thereby lowering the concentration of free Mg^{++} in the assay and reducing AC activity by an indirect effect (see fig. 23). 5-HT did not increase the inhibition produced by Gpp(NH)p (fig. 20B), yet 5-HT increased basal AC activity in the presence of Gpp(NH)p (fig. 21B). A possible explanation for the difference may be that G_i, the regulatory protein mediating inhibition of AC activity, hydrolyzes GTP 10 times more rapidly than G_s (Birnbaumer et al., 1985). Therefore, GTP remains bound long enough to activate G_s but not G_i. Receptor stimulation probably shortens the rate limiting step in the sequence of events from GTP binding to a reduced rate of adenylyl cyclase activity. The nature of the rate limiting step for inhibition of AC has not yet been established with purified components of the AC system, but may be the release of GDP from G_i (Birnbaumer et al., 1985). Such a mechanism

would explain why GTP is ineffective in inhibiting AC, but Gpp(NH)p, which is not hydrolyzed to GDP, is not affected by receptor stimulation. The concentration of GTP in intact cells is approximately 100 µM, making the effect of 5-HT with respect to GTP (fig. 20A) maximal under physiological conditions.

Inhibition and stimulation of AC activity also are characteristically different with respect to the effects of Mg^{++}. 5-HT decreased the EC_{50} of Mg^{++} (fig. 22) in stimulating basal AC activity, from about 6 to 2 mM Mg^{++}. Since the concentration of free Mg^{++} in cells is approximately 0.5 mM (Iyengar and Birnbaumer, 1982), this effect of 5-HT should produce a dramatic effect in the extent of activation by Mg^{++}. The effect of 5-HT in reducing the EC_{50} of Mg^{++} may be even more pronounced than is apparent from these data, since 5-HT may stimulate only a part of the total available regulatory proteins, as evidenced by the finding that 5-HT and histamine stimulation is additive in guinea pig hippocampal membranes (Shenker et al., 1983). Iyengar and Birnbaumer (1982) demonstrated that the function of the glucagon receptor is to accelerate the activation of G_s by increasing its affinity for Mg^{++}. Glucagon receptors act as "Mg^{++} switches", allowing the ambient concentration of Mg^{++} to shorten the activation time of the G_s protein such that GTP stimulated its activation. This does not preclude an effect of the receptor on exchange of nucleotides independent of the effect of the receptor on Mg^{++}. In contrast to stimulation, inhibition requires much lower concentrations of free Mg^{++}, as evidenced by the fact that inhibition by 5-HT was maximal at low (micromolar) concentrations of Mg^{++} (fig. 23). The low requirement of inhibition for Mg^{++} compared to the millimolar requirement for stimulation is consistent with the low (micromolar) requirement of G_i for activation by Mg^{++} (Hildebrandt and Birnbaumer,

1983).

In conclusion, the results from this study with respect to guanyl nucleotides and Mg^{++} support the contention that the inhibitory effect is mediated by a GTP-binding protein resembling G_i and the stimulation by G_s. This conclusion is based on the micromolar EC_{50} values for both inhibition and stimulation by GTP and the low requirement of inhibition for Mg^{++} (micromolar) compared to stimulation (millimolar). Further support for the identity of the inhibitory regulatory protein as G_i comes from the results with pertussis toxin (see below).

IV. 6 Regulation of 5-HT receptors by exposure to 5-HT

Regulation of 5-HT_{1A} receptors in the presence of a reduced concentration of 5-HT in vivo was studied by administration of the 5-HT-depleting drug reserpine. Membranes from reserpinized guinea pigs expressed as much as a three-fold reduction in the EC_{50} value of 5-HT in inhibiting AC activity (fig. 24), suggesting that 5-HT_{1A} receptors rapidly ($<$ 24 hrs) adapt to a decrease in 5-HT input by an increase in sensitivity to 5-HT. Whether this increased sensitivity results from an increased number of receptors or from an increased coupling efficiency of receptors with their effectors cannot be ascertained from these experiments. For the partial agonist buspirone, 5-HT depletion resulted in either a decrease in the EC_{50} or an increase in the intrinsic activity of buspirone (data not shown), suggesting that buspirone may act as a full agonist if the 5-HT input to a tissue is reduced or if the concentration of 5-HT_{1A} receptors in a tissue is high.

In homogenates from reserpinized guinea pigs, the maximal stimulatory effect of 5-HT on basal AC activity was doubled with no change in the EC_{50} parameter (Shenker et al., 1983; fig. 25). Treatment of guinea pigs with the 5-HT precursor, 5-HTP, a treatment shown to increase 5-HT levels in brain (Luscombe et al., 1983), produces the opposite effect on the magnitude of 5-HT stimulation of AC. In membranes from 5-HTP-treated animals, the stimulation by 5-HT of AC was reduced by nearly 50% compared to paired controls, and no change was measured in the mean percentage of stimulation by histamine, indicating that the reduction in stimulation by 5-HT did not result from an increase in 5-HT content in the assay. Therefore, 5-HT receptors mediating stimulation of AC rapidly adapt to reduced 5-HT input. The magnitude of the stimulatory effect appears to be receptor-limited since reserpinization results in an increased magnitude of the 5-HT response, whereas the magnitude of the inhibitory effect is effector-limited. At a fixed concentration of 5-HT near the EC_{50} of the receptors, the effect of reducing the EC_{50} of 5-HT is comparable to doubling the magnitude of the response. For example, at a concentration of 5-HT of 25 nM, the effect of reducing the EC_{50} from 50 to 15 nM would result in an increase from 33% of maximum to 83% of maximum.

Incubation of membranes with 5-HT reduces the stimulation by 5-HT itself (fig. 26). In contrast, histamine-stimulated activity is a constant fold-stimulation of the basal activity. The decrease in 5-HT-stimulated activity was not a result of degradation of 5-HT since addition of 5-HT during the time of the experiment did not restore the responsiveness of the tissue. The decrease in 5-HT-stimulated activity is not result of a reduction in 5-HT receptors during the time of incubation since incubation of the tissue without 5-HT does not reduce the responsiveness of the tissue to a

subsequent challenge with 5-HT. Therefore, incubation of guinea pig hippocampal homogenates with 5-HT reduces the responsiveness of the homogenates to 5-HT. The concentration-response curve of 5-HT for this reduction (fig. 27) resembles the 5-HT_{1A} concentration-response curve for inhibition. This suggests that the "desensitization" of the 5-HT stimulation is actually a manifestation of inhibition by 5-HT. In addition, "desensitization" by 5-HT required GTP and Mg^{++}, supporting the contention that desensitization resulted from activation of an inhibitory pathway by 5-HT (fig. 28A). These results suggest that inhibitory and stimulatory 5-HT receptors may be functionally coupled in the guinea pig hippocampus, much like α_2 and ß-adrenergic receptors in other tissues.

In membranes from 5-HTP-treated animals, incubation with 5-HT further reduced the stimulation by 5-HT (fig. 28B). Therefore, 5-HTP treatment and incubation of homogenates with 5-HT may reduce the responsiveness of 5-HT receptors positively coupled to AC by different mechanisms. A reasonable hypothesis to explain these results is that 5-HTP treatment reduces sensitivity of the tissue to 5-HT by desensitizing 5-HT receptors positively coupled to AC, whereas incubation of homogenates with 5-HT reduces stimulation by activating inhibitory (5-HT_{1A}) receptors.

IV. 7 Physiological roles for 5-HT receptors coupled to adenylyl cyclase

Pertussis toxin (PT) is an enzyme that inhibits coupling of G_i to receptors (Ui et al., 1984). Since incubation of guinea pig hippocampal membranes with PT reduced the percentage of inhibition by 5-HT (fig. 29),

inhibition of forskolin-stimulated AC activity by 5-HT is mediated by a PT-sensitive substrate. Incubation of the membranes for longer periods of time resulted in a complete loss in activity in both PT-treated and control membranes, making this approach not useful in examing the effects of PT. Pretreatment of rats in vivo with PT reduces or abolishes 5-HT receptor-mediated inhibition of AC (fig. 30A, 31). The in vivo approach allows incubation of the tissue with PT for longer (2 to 3 days) periods of time.

In addition to its effect on 5-HT-mediated inhibition of AC activity, PT reduced the decrease in population spike amplitude (PSA) elicited by 10 µM 5-HT (fig. 30). The PSA is an extracellular field potential measured by recording the field potential in the CA1 region of the hippocampal slice after stimulating the stratum radiatum (Beck and Goldfarb, 1985). The potential is proportional to the number of discharging pyramidal cells in the region of the recording electrode. 5-HT reduces the evoked field potential, indicating it can reduce the probability of pyramidal cell discharge. The reduction in the decrease in PSA and inhibition of AC by 5-HT are proportional and show nearly identical dose-dependencies with respect to PT, (fig. 32,33), suggesting that both may be mediated by a common regulatory protein, presumably G_i.

The relationship between inhibiton of AC and the decrease in PSA is not yet clear. Three logical possibilities exist: (1) a reduction in cAMP could cause the decrease in PSA, (2) both the decrease in PSA and the inhibition of AC could occur independently of each other, or (3) the inhibition of AC could act in concert with another 5-HT_{1A} receptor-mediated PT-sensitive effect to decrease PSA. There is evidence suggesting that changes in cAMP affect the PSA in the rat hippocampal slice. Agents known to increase or mimic cAMP in the hippocampus, including ß-adrenergic receptor agonists and

cAMP analogs (Mueller et al., 1981), H_2 receptor agonists (Haas and Greene, 1986) and forskolin (Lin-Liu et al., 1984) all increase PSA in the CA1 region of the rat hippocampal slice. Conversely, agents that decrease cAMP, including 5-HT_{1A} agonists, α_2-adrenergic receptor agonists (Sabol and Nirenberg, 1979) and $GABA_B$ agonists (Wojik and Neff, 1984) all decrease PSA in the CA1 region of the rat hippocampal slice (Mueller et al., 1981; Ault and Nadler, 1983).

Alternatively, the 5-HT_{1A}-mediated decrease in PSA occurs independently of the change in cAMP levels. Numerous examples have been reported where a regulatory protein mediates separate effects within the same cell. For example, a PT-sensitive G protein, activated by angiotensin, mobilizes Ca^{++} in hepatocytes independently of its inhibitory effect on cAMP levels (Johnson et al., 1986). A PT-sensitive regulatory protein couples muscarinic receptors directly to K^+ channels in cardiac muscle cells (Martin et al., 1985) independently of the inhibition of AC in those cells. In a recent abstract, Andrade et al. (1986) reported that 8-bromo-cAMP did not affect the 5-HT_{1A} mediated hyperpolarization of rat hippocampal pyramidal cells. These authors suggested that the 5-HT response was mediated directly by a PT-sensitive protein directly coupled to a K^+ channel.

Therefore, there is evidence for both cAMP-dependent and cAMP-independent components to the PT-sensitive 5-HT_{1A} receptor-mediated decrease in PSA. The decrease in pyramidal cell firing may result from directly opening a K^+ channel (hyperpolarizing the cells). However, in cells receiving inputs that increase cAMP and thereby close Ca^{++}-activated K^+ channels (Madison and Nicoll, 1982; Haas and Konnerth, 1983), 5-HT_{1A} receptors may decrease formation of cAMP and thereby contribute to the overall increase in K^+ conductance. The possible interaction of 5-HT_{1A}

receptors with the adenylyl cyclase system and K^+ channels is shown in fig. 34. This hypothesis may be true for other receptors coupled to G_i. It is interesting to note that receptors which, through PT-sensitive mechanisms, open potassium conductances (5-HT_{1A} and $GABA_B$, Andrade et al., 1986; α_2 and opiate, Aghajanian and Wang, 1986; muscarinic, Martin et al., 1985; adenosine A_1, Trussel and Jackson, 1986) are also associated with inhibition of AC.

The opposing actions of 5-HT_{1A} and ß-adrenergic receptors on cAMP formation and, possibly, the opening of K^+ conductances, may account for the observation that some antidepressants decrease the sensitivity of ß-adrenergic pathways (Wolfe et al., 1978) and others increase the sensitivity of serotonergic ones (De Montigny and Aghajanian, 1978; Blier et al., 1984). If all antidepressants ultimately affect a common mediator of both pathways, than desensitization of ß-adrenergic input or sensitization of serotonergic ones may have a final equivalent effect on an intact tissue.

IV. 8 Summary and conclusions

5-HT_{1A} receptors mediate an inhibition of forskolin-stimulated AC activity in guinea pig and rat hippocampal membranes and rat cerebral cortical membranes. Inhibition by 5-HT_{1A} receptors is probably mediated by G_i as evidenced by the pertussis toxin sensitivity of the response and the requirement for micromolar concentrations of GTP and Mg^{++}. The magnitudes of the inhibition of AC and decrease in PSA by 5-HT are correlated and show similar responsiveness to PT-treatment, suggesting

that both may be mediated the same PT-sensitive substrate. 5-HT_{1A} receptors increase their sensitivity to 5-HT after depletion of 5-HT in vivo. Stimulation of AC activity in guinea pig hippocampal membranes is mediated predominantly by a distinct receptor that is apparently homologous with the receptor that stimulates AC activity in infant rat collicular membranes. Stimulation of AC activity by 5-HT requires millimolar concentrations of Mg^{++}, a characteristic of G_s proteins. Stimulatory receptors also adapt to changes in exposure to 5-HT in vivo. The 5-HT_{1A} and R_L receptors are functionally coupled in an opposing manner to adenylyl cyclase, at least in membranes prepared from guinea pig hipppocampi.

V. FIGURES

Figure 1 : Information flow through G protein systems. Note that some of the above examples are speculative (e.g. phospholipase C has not been shown to be directly coupled to a G protein).

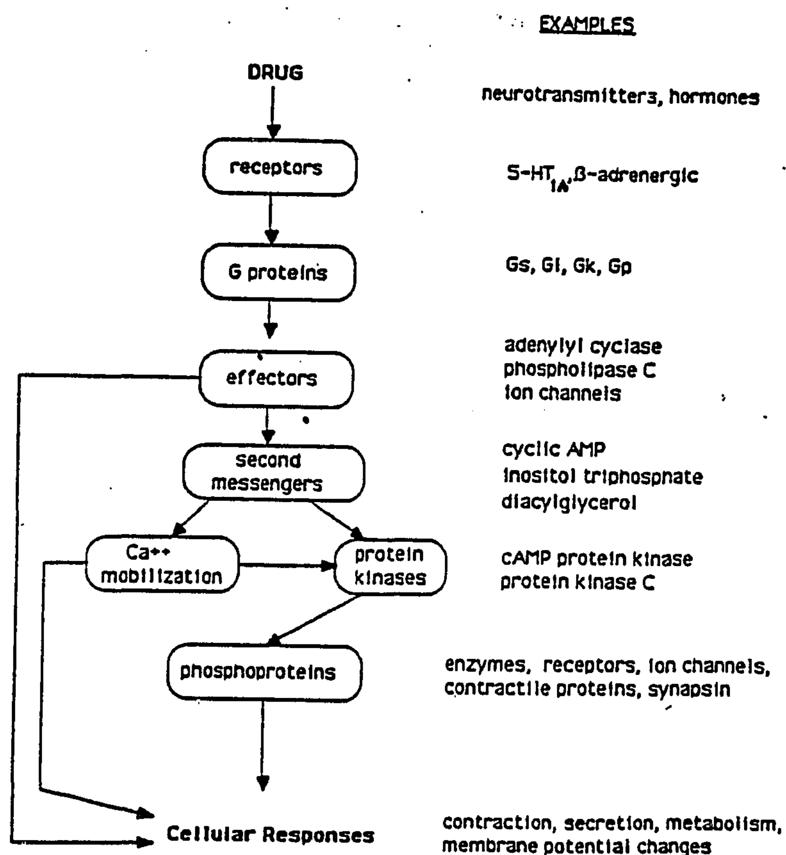


Figure 2 : Forskolin-stimulated adenylyl cyclase activity.
Forskolin (o) stimulated AC activity in guinea pig hippocampal membranes from 44 to an E_{max} of 240 pmols/mg/min., and with an EC_{50} of 0.4 µM. In the presence of 10 µM 5-HT (•), basal activity was stimulated by 85% to 81 pmols/mg/min. and the E_{max} was inhibited by 32% to 164 pmols/mg/min. Data points are the means of three measurements and the points preceding the break in the curve represent activity in the absence of forskolin. Equation 5 was fit to the data with an R^2 value of 0.99.

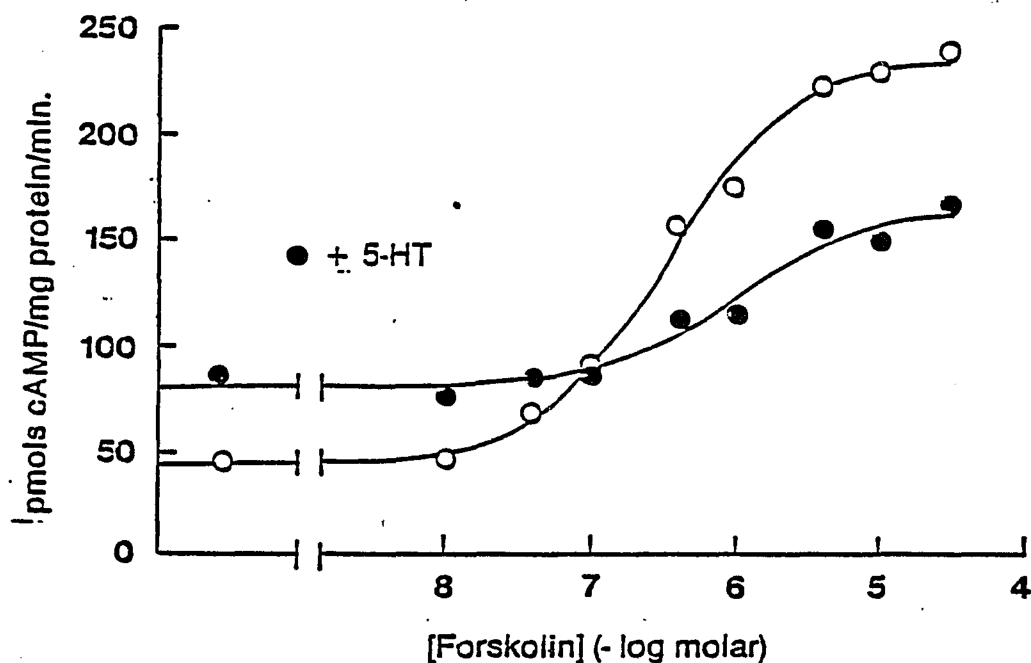


Figure 3 : Effect of time of incubation and membrane concentration on forskolin-stimulated AC activity. Forskolin (10μM) stimulated AC activity in guinea pig hippocampal membranes. In the absence (o) and presence (•) of 5-HT (10 μM), stimulation was linear with respect to time for at least 15 minutes (A) and with respect to membrane concentration for up to 40 μg per assay (B). Data points are means of three measurements.

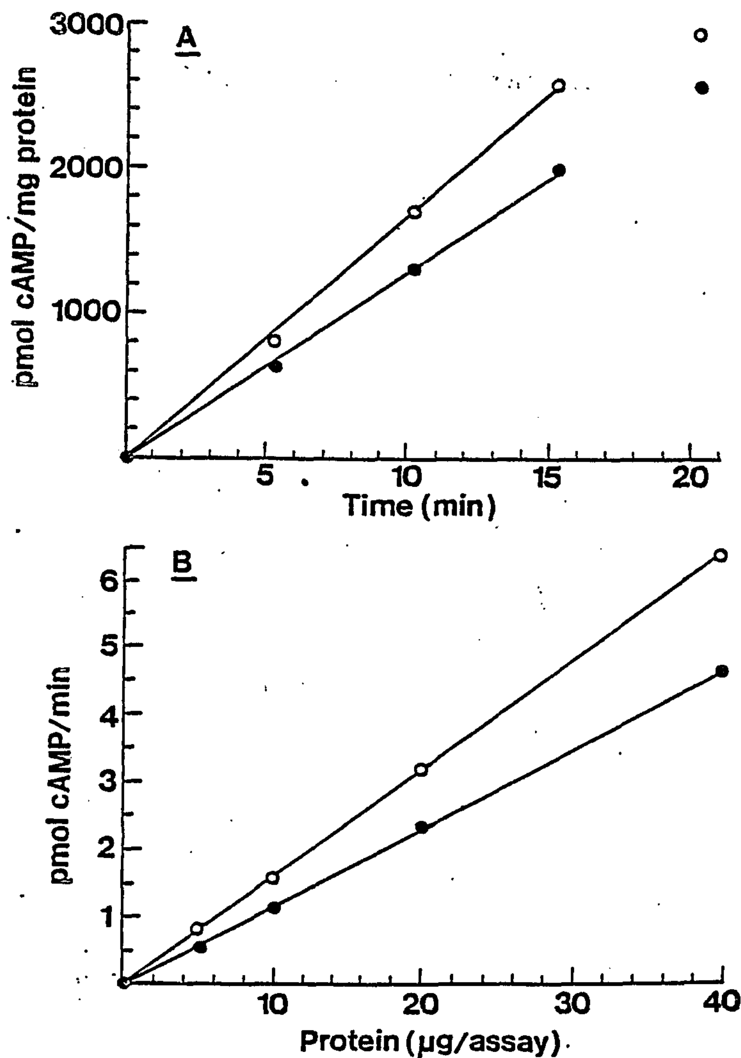


Figure 4 : Inhibition of adenylyl cyclase by 5-HT.
(A) Forskolin-stimulated AC activity in guinea pig hippocampal membranes was inhibited by 5-HT. Data points are means of three measurements and the average SEM was 2 pmols. (B) Concentration-reponse curves with a slope factor (equation 3; dashed line, n = 1.4, $R^2 = 0.98$) and without a slope factor (equation 1; solid line, n=1, $R^2 = 0.97$) were fitted to the data. 5-HT inhibited forskolin-stimulated activity by 26% from 185 to 136 pmols/mg/min. and with an EC_{50} value of 33 nM. Points preceding the break in the curve represent activity measured in the absence of 5-HT and were included in the fit of equation 5 to the data.

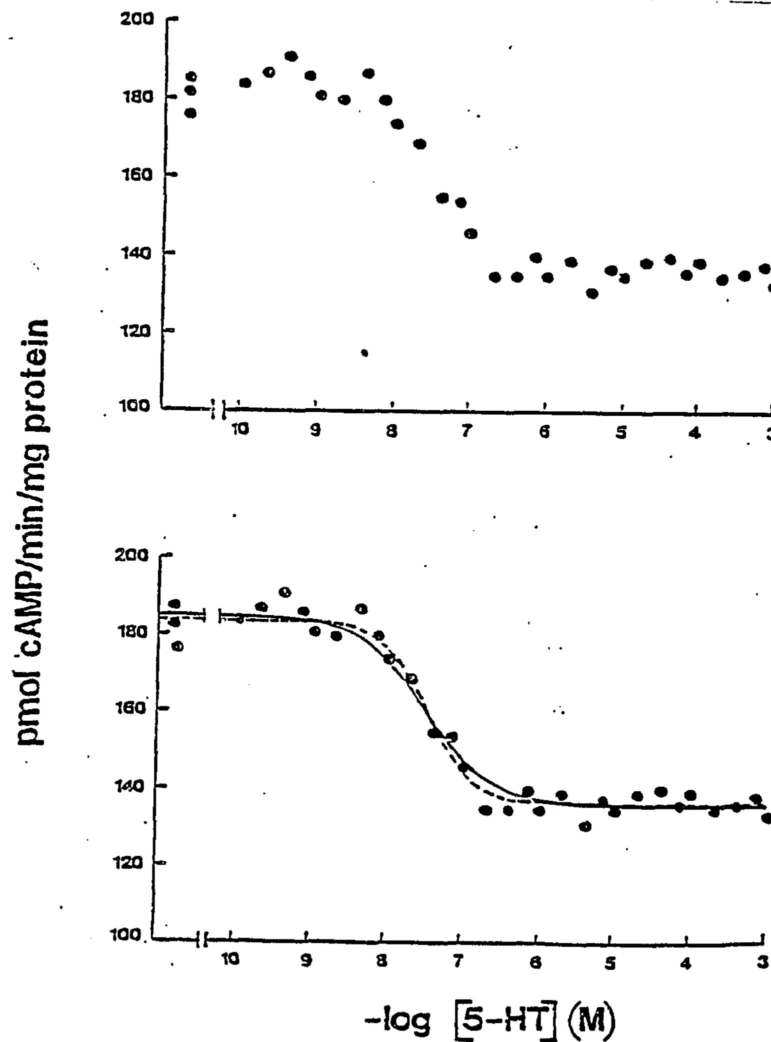


Figure 5 : Response of guinea pig hippocampal membranes to PAT, RU 24969 and buspirone. Concentration-response data were normalized to 100. Forskolin-stimulated activity in each experiment was: 92 pmol cAMP/mg/min (PAT), 118 (RU 24969) and 87 (buspirone). EC_{50} values of each agonst were: 20 nM (PAT, $R^2 = 0.95$), 52 nM (RU 24969, $R^2 = 0.98$) and184 nM (buspirone, $R^2 = 0.91$). Data points are means of three measurements. The points preceding the break in the curve represent activity measured in the absence of agonist and were included in the fit of equation 5 to the data.

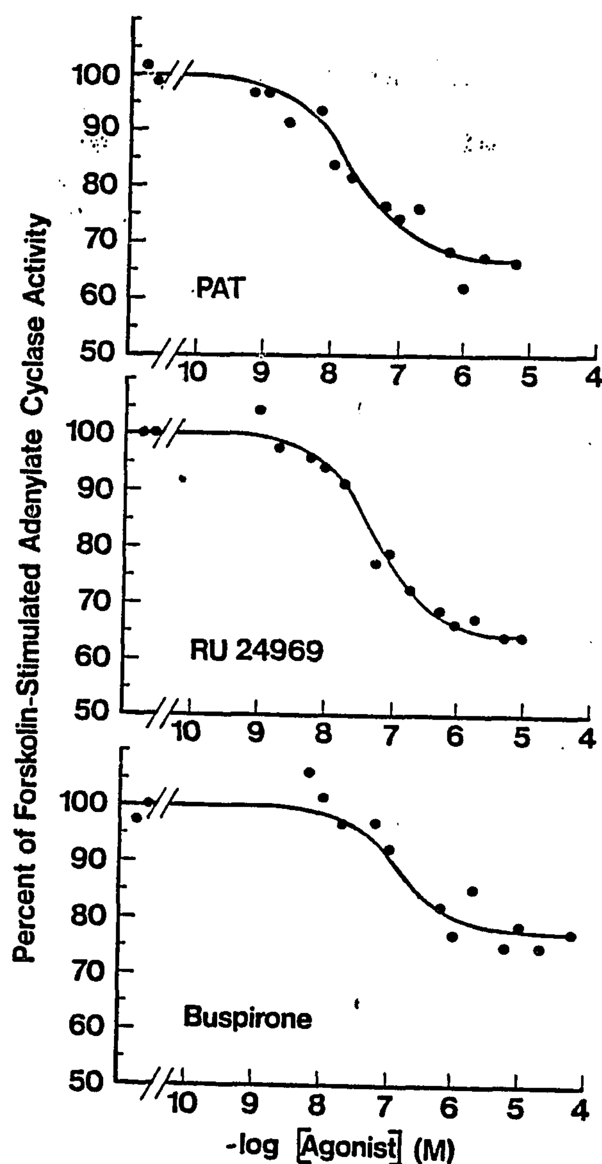


Figure 6 : Response of guinea pig membranes to tryptamine derivatives. Concentration-response curves to 5-CT (A) and 5-methoxytryptaminef (5-MT) (B) were normalized to 100. Forskolin-stimulated activity in the absence of agonist was 167 (5-CT) and 129 (5-MT). EC_{50} values were: 6.8 nM (5-CT, $R^2 = 0.99$) and 63 nM (5-MT, $R^2 = 0.96$). The points preceding the break in the curve represents activity measured in the absence of agonist and were included in the fit of equation 5 to the data. Data points are means of three measurements.

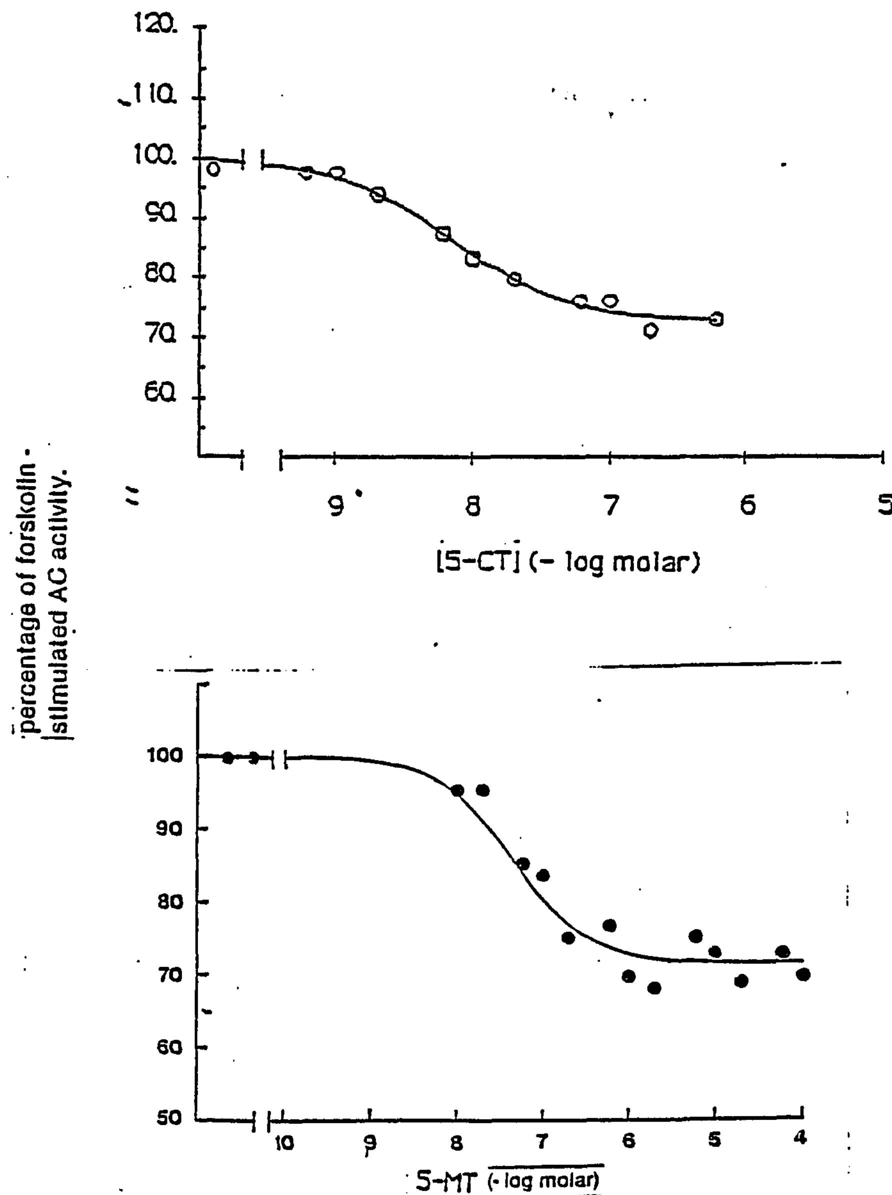


Figure 7 : Response of guinea pig membranes to piperazine derivatives. Concentration-response curves to PAPP (A) and TFMPP (B) were normalized to 100. Forskolin-stimulated activity in the absence of agonist was 190 (PAPP) and 182 (TFMPP). PAPP inhibited AC activity by 30% with an EC_{50} value of 15 nM (R^2 = 0.99); TFMPP inhibited activity by 27% with an EC_{50} value of 1.9 µM (R^2 = 0.92). The points preceding the break in the curve represent activity measured in the absence of agonist and were included in the fit of equation 5 to the data. Data points are means of three measurements and the average SEM was 2.7 pmols.

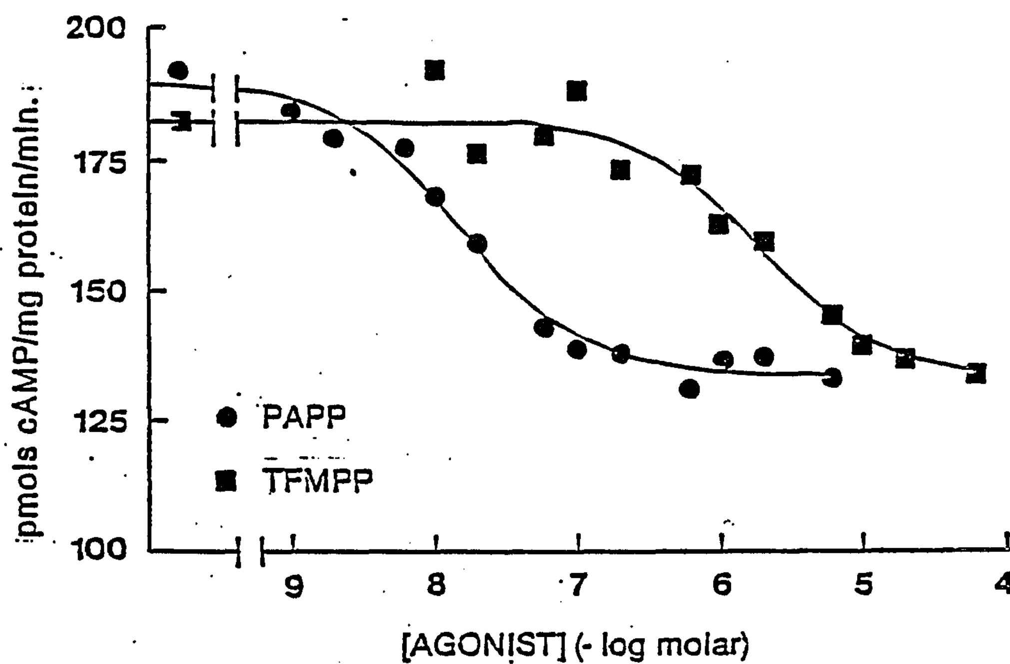


Figure 8 : Response of guinea pig membranes to ergot derivatives. Concentration-response curves were normalized to 100. Forskolin-stimulated activity was 92 (LSD), 115 (methysergide, MS) and 80 (metrgoline, MET) respectively. The EC_{50} values were: 14 nM (LSD, R^2 = 0.94), 322 nM (MS, R^2 = 0.0.97) and 102 nM (MET, R^2 = 0.96). The points preceding the break in the curve represents activity measured in the absence of agonist and were included in the fit of equation 5 to the data. Data points are means of three measurements.

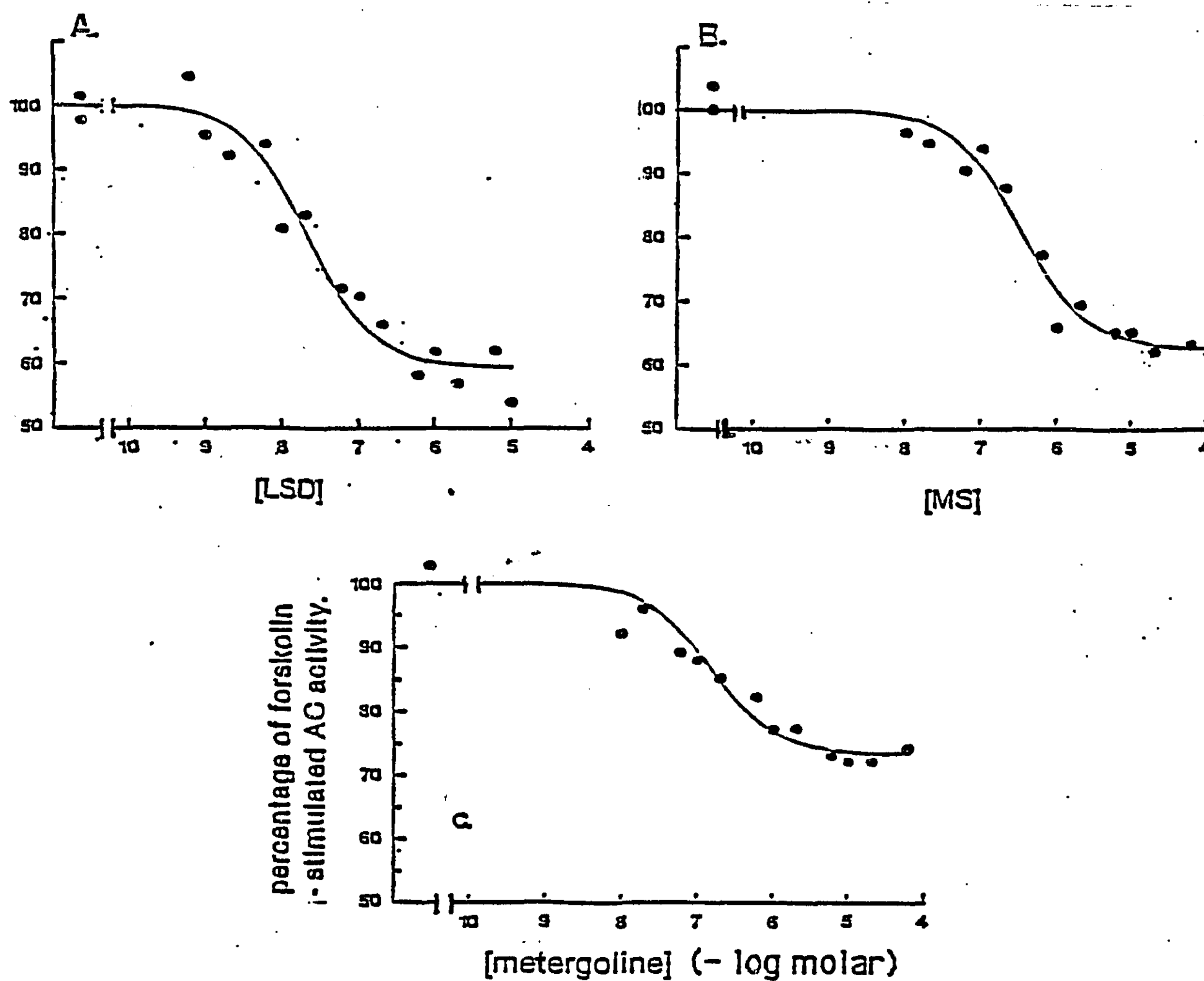


Figure 9 : Response of rat hippocampal membranes to lisuride, PAT and buspirone. Concentration-response data were normalized to 100. Forskolin-stimulated activity in the absence of agonist was: 215 (lisuride, ●), 379 (PAT, ■) and 140 (buspirone, ▲). EC_{50} values were: 6.0 nM (lisuride, $R^2 = 0.98$), 32 nM (PAT, $R^2 = 0.98$) and 76 nM (buspirone, $R^2 = 0.95$). Data points are the means of three measurements.

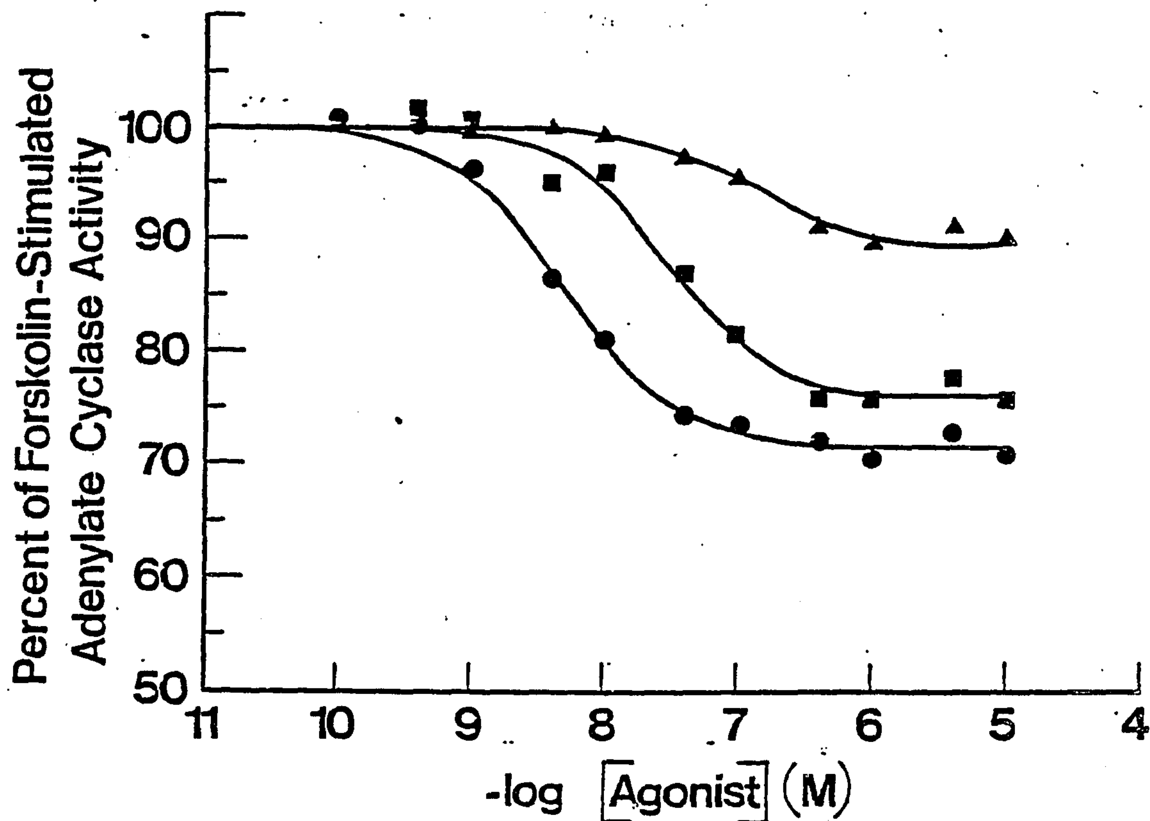


Figure 10 : Antagonism by methiothepin and spiperone. Data were normalized to 100. Methiothepin, at concentrations of 0.4 (●) and 4 µM (▲), increased the EC_{50} value of 5-CT to 0.35 and 3.5 µM, respectively, with no decrease in the maximal percentage of inhibition by the agonist (35% and 32%). AC activity in the absence of 5-CT was 164 and 170 pmols/mg/min., respectively. Spiperone, at concentrations of 4 (●) and 10 µM (▲), increased the EC_{50} of 5-CT to 0.66 and 1.8 µM, respectively, with maximal percentages of inhibition of 35% and 31%. AC activity in the absence of 5-CT was 190 and 191 pmols/mg/min. A concentration-response curve to 5-CT alone (o) is included for comparison.

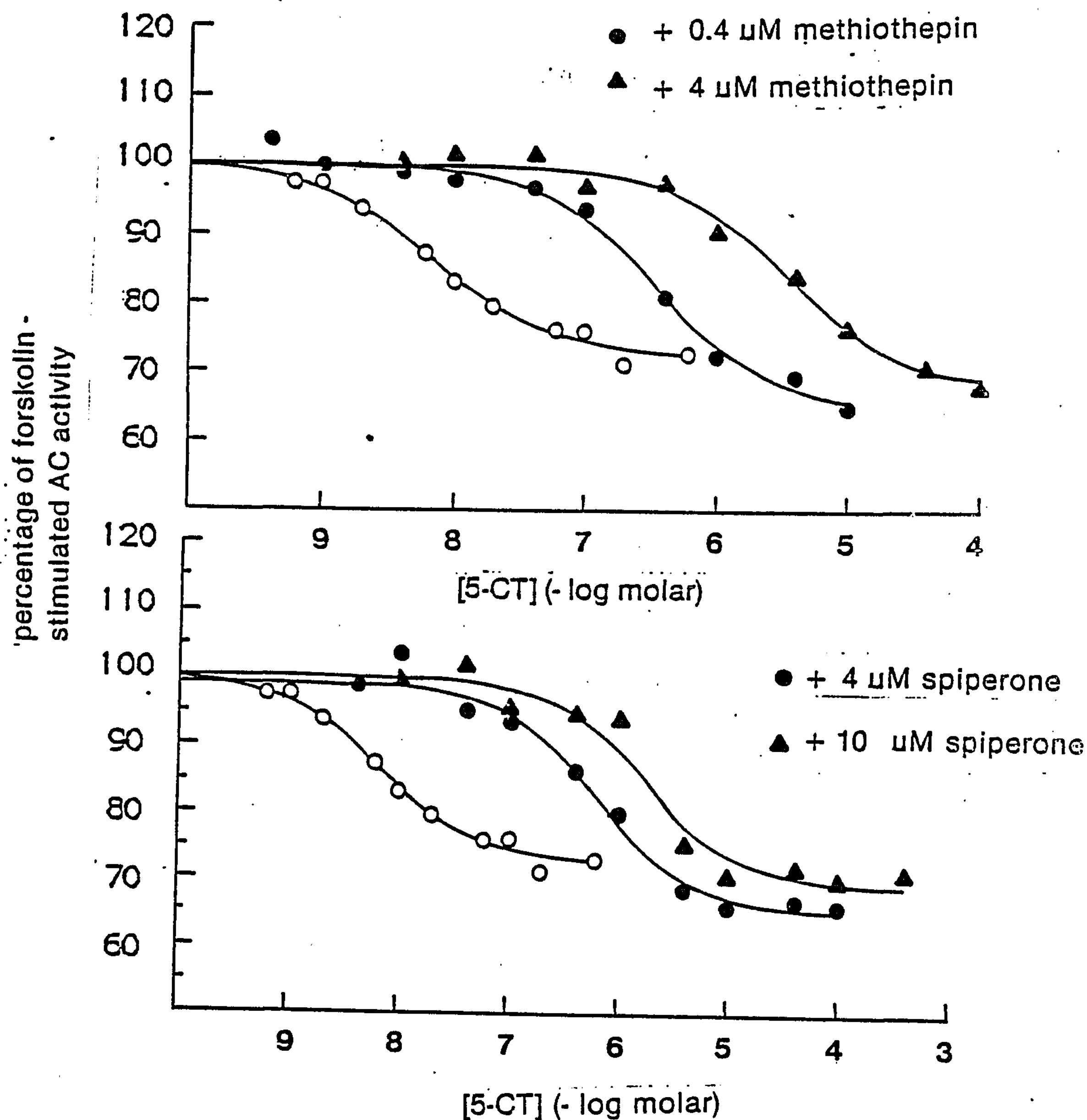


Figure 11 : Schild plots of the antagonism by methiothepin and spiperone. Schild plots of the EC_{50} values of 5-CT in the presence of various concentrations of antagonists yielded a K_b value for methiothepin of 13 ± 1 nM (R^2 = 0.92; unit slope) and a K_b value for spiperone of 26 ± 1 nM (R^2 = 0.96; unit slope). The error was calculated from the antilog of the standard deviation of the regression multiplied by the K_b value for each antagonist.

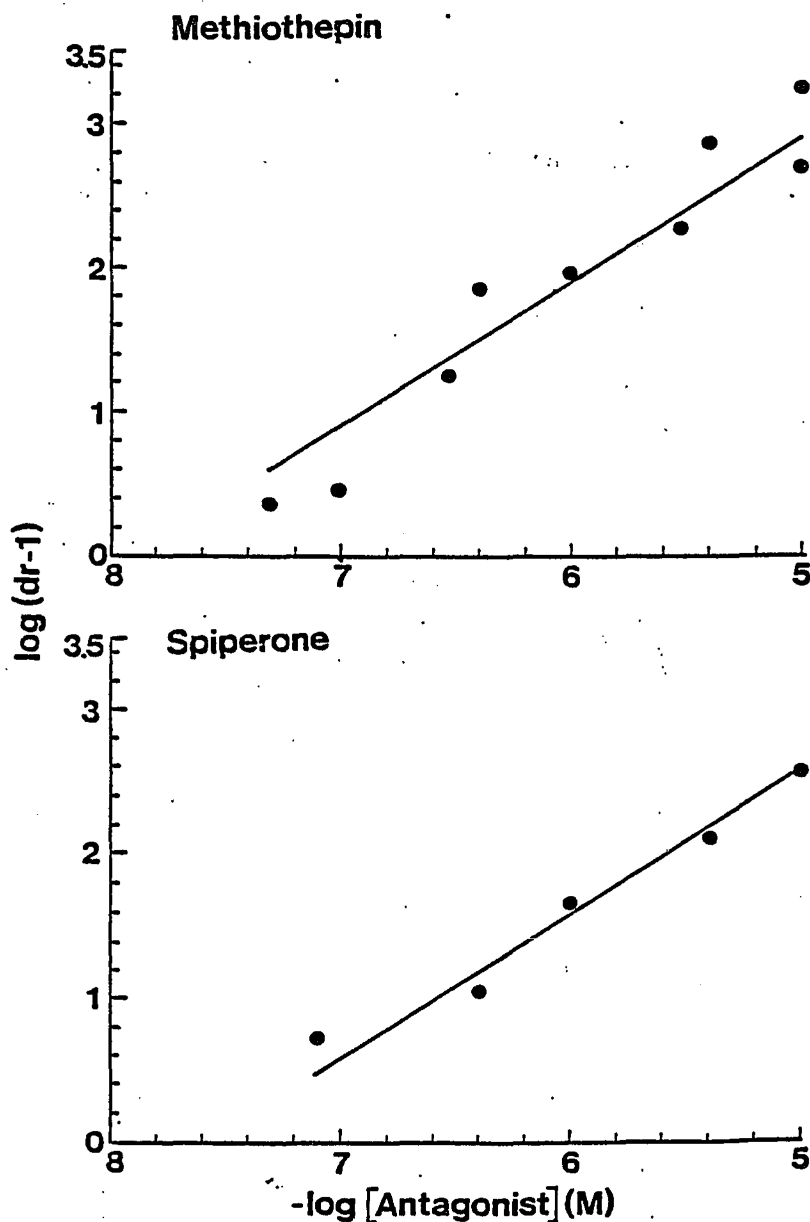


Figure 12 : Antagonism by (-)pindolol of the inhibition by 5-HT of AC activity in rat hippocampal membranes. (A) Data were normalized to 100. (-)Pindolol, at concentrations of 0.5 (•) and 5 µM (▲), increased the EC_{50} value of 5-HT to 0.88 and 12 µM, respectively, with a maximal percentage of inhibition by 5-HT of 22% at each concentration of (-)pindolol. AC activity in the absence of 5-HT was 157 and 151 pmols/mg/min. A representative experiment with 5-HT alone (o) is included for comparison (EC_{50} = 51 nM, maximal percentage of inhibition = 26%). (B) A Schild plot (R^2 = 0.96; unit slope) of the EC_{50} values of 5-HT at four different concentrations of pindolol yielded a K_b value of 40 ± 4 nM. S.D. was calculated as the antilog of the standard deviation of the regression multiplied by the K_b value of pindolol.

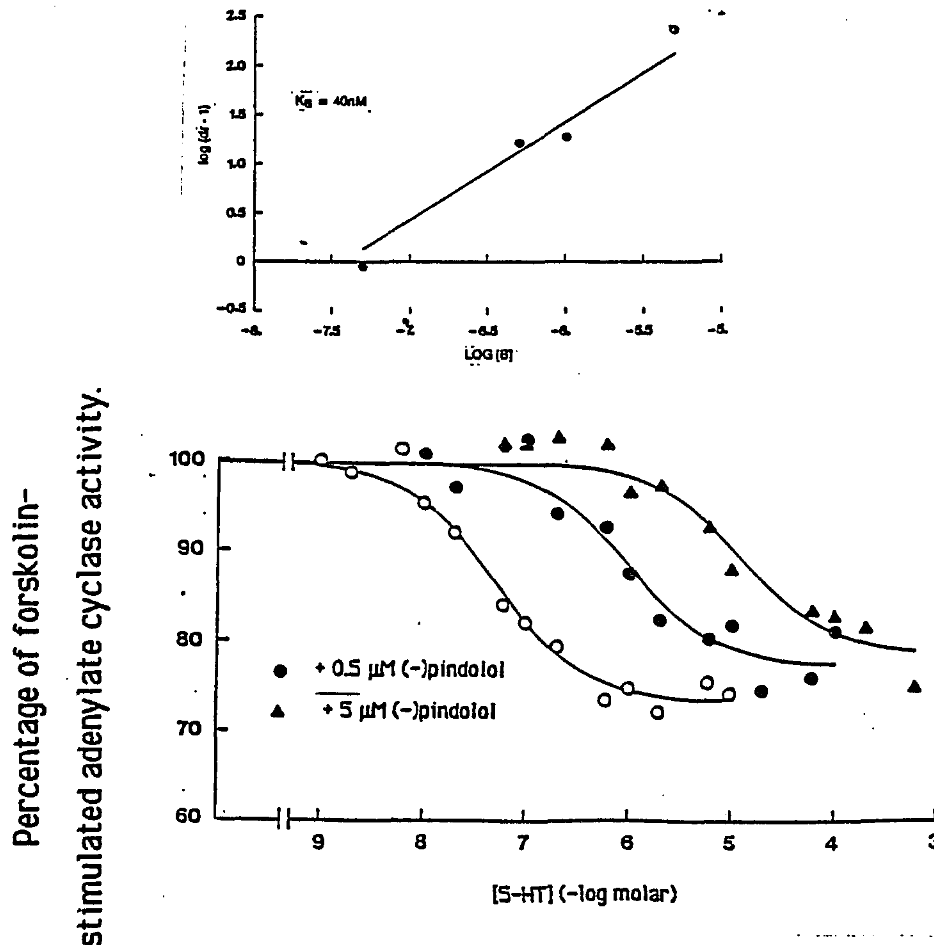


Figure 13 : Lack of antagonism by ketanserin. In the absence of ketanserin, 5-CT (o) inhibited AC activity in guinea pig membranes by 25% from 68 to 51 pmols/mg/min. with an EC_{50} of 8 nM. In the presence of 1 μM ketanserin, 5-CT (•) inhibited activity from 69 to 52 pmols/mg/min. with an EC_{50} of 7 nM.

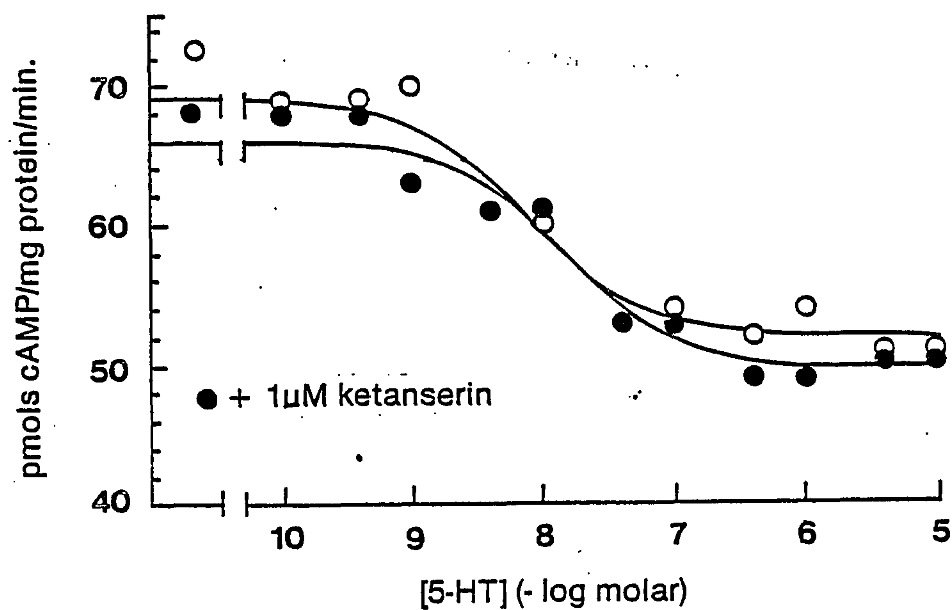


Figure 14: Concentration-response data with membranes from rat cerebral cortex. (A) 5-CT (•) inhibited forskolin-stimulated AC activity in rat cerebral cortex membranes by 17% from 68.0 to 56.4 pmols/mg/min. with an EC_{50} value of 6.0 nM (R^2 = 0.94). Buspirone (■) inhibited activity by 9.6% from 62.3 to 56.3 pmols/mg/min. with an EC_{50} of 450 nM (R^2 = 0.77). (B) Data were normalized to 100. Buspirone (10 µM) increased the EC_{50} of 5-CT from 30 (o) to 200 (•) nM and decreased the percentage of inhibition by 5-CT from 21 to 17%. AC activity in the absence of 5-CT was 140 and 126 pmols/mg/min. Data points are the means of three measurements with an average SEM of 1.1 pmol.

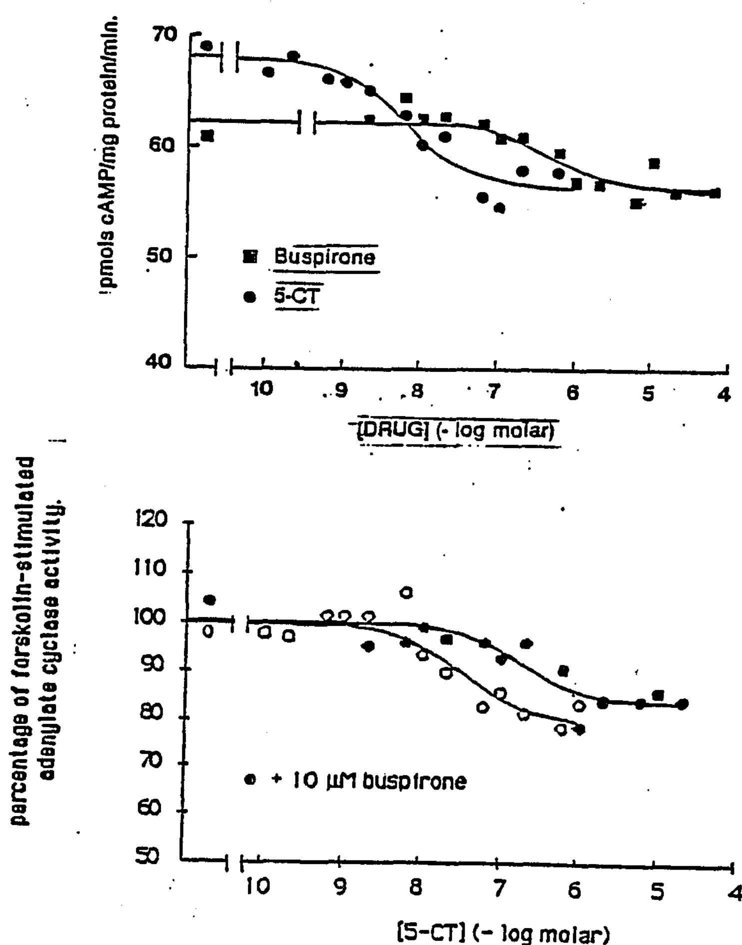
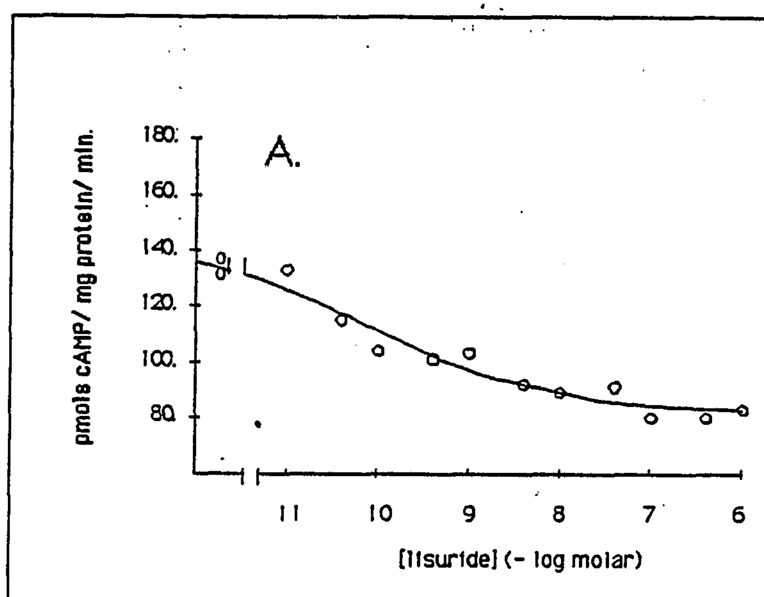


Figure 15 : Anomalous inhibition by lisuride in guinea pig membranes. (A) Lisuride inhibited forskolin-stimulated AC activity in guinea pig membranes from 144 to 82 pmols/mg/min. with an EC_{50} value of 80 pM and a slope factor of 0.44 ($R^2 = 0.96$) (parameter estimates were obtained by a fit of equation 5 to the data). (B) With membranes from a different animal, in the presence of 1 μM spiperone, lisuride inhibited activity by 32% from 151 to 103 pmols/mg/min. with an EC_{50} value of 450 nM (no slope factor used, $R^2 = 0.84$). Data points are means of three measurements with an average SEM of 8.1 pmols. Points preceding the break in the curve rrepresent activity in the absence of 5-HT and were included in the fit of the equation to the data.



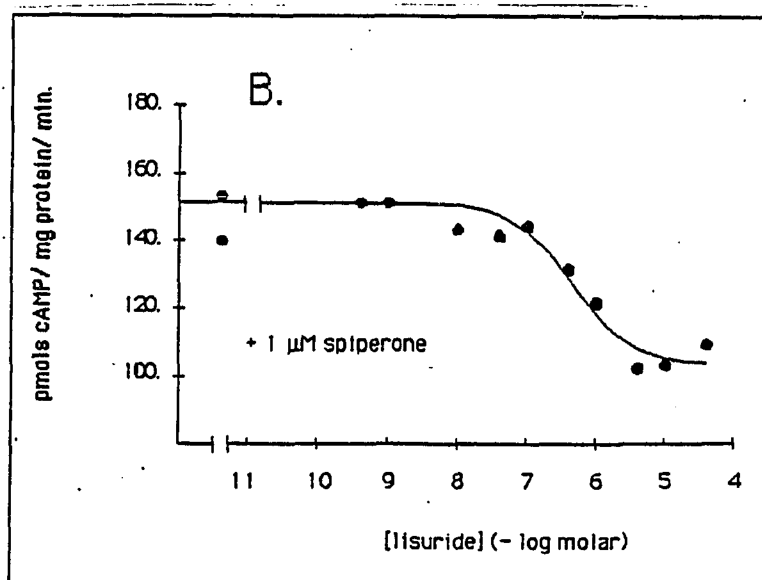


Figure 16 : Stimulation of basal AC activity by 5-HT and PAT in guinea pig hippocampal membranes. 5-HT (▲) stimulated AC activity in the crude membrane preparation from 22 to 35 pmols/mg/min. with an EC_{50} of 220 nM. PAT stimulated activity from 21 to 24 pmols/mg/min with an EC_{50} of 570 nM. The data were fit with a one-site equation (without a slope factor). R^2 values were 0.98 (5-HT) and 0.73 (PAT). Points represent means of three measurements with an average SEM of 1.0 pmols/mg/min.

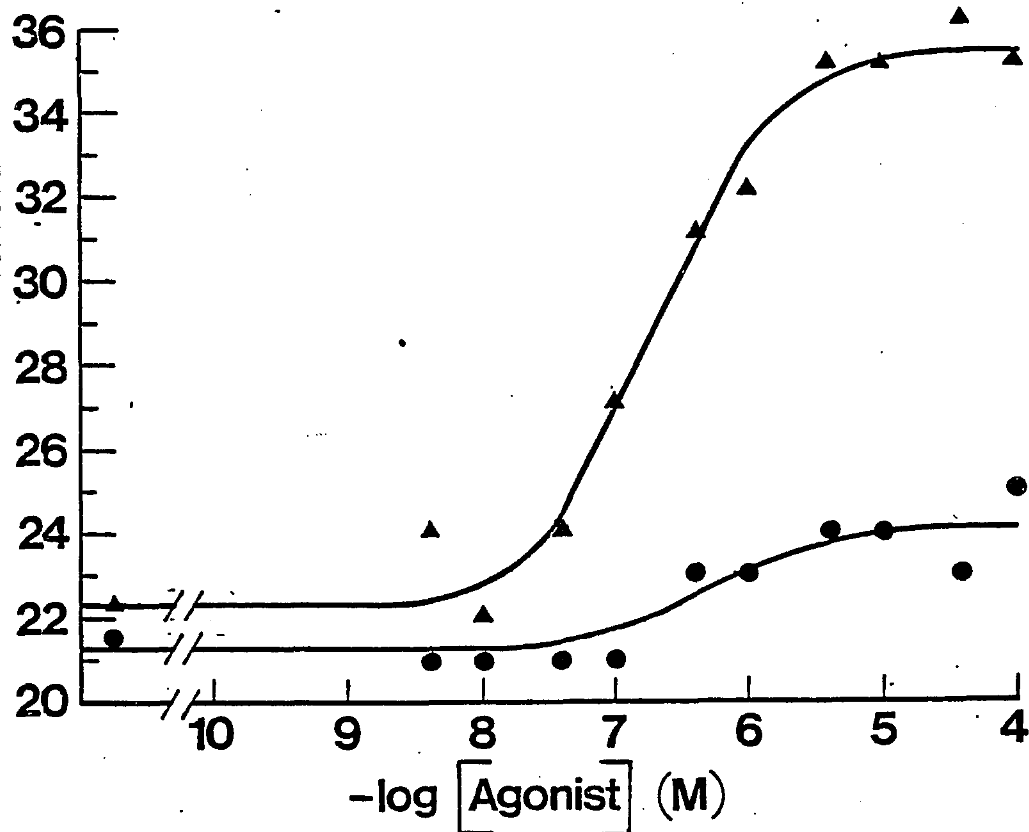


Figure 17 : Effect of membrane concentration on 5-CT stimulation of AC activity. 5-CT (•), at concentrations of 1 nM to 1 µM, produced no observable increase in basal AC activity with guinea pig hippocampal membranes when the concentration of membrane protein was 32 µg per assay. At a membrane concentration of 46 µg, 5-CT (o) increased AC activity by 54% from 13.6 to 21 pmols/mg/min. with an EC_{50} value of 6 nM (R^2 = 0.91). Data points are means of three measurements with an average SEM of 0.60.

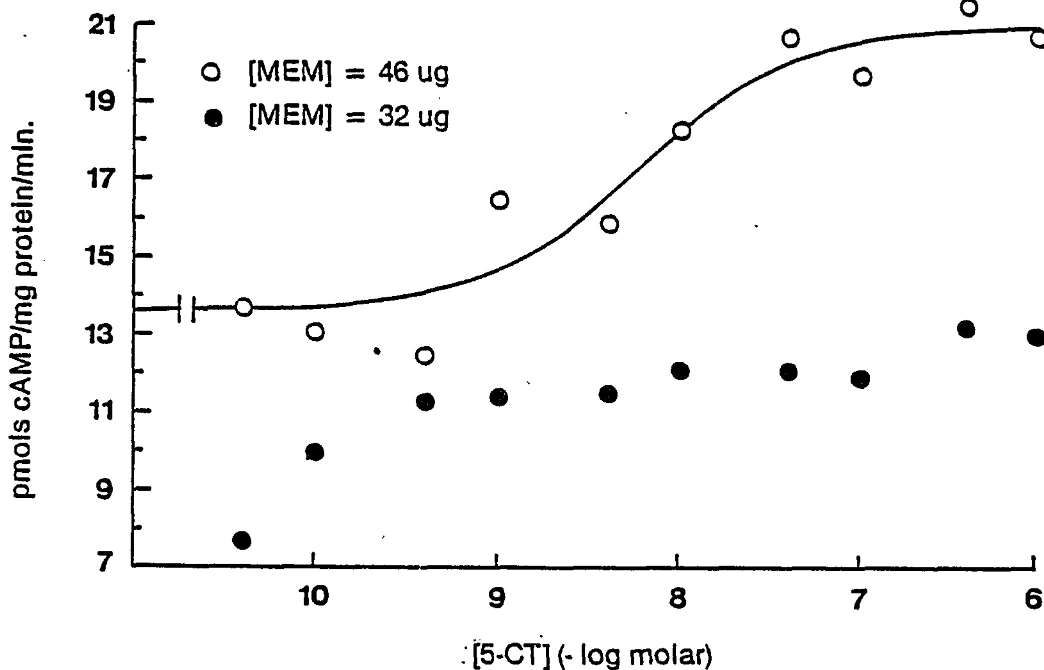


Figure 18 : Effect of histamine on forskolin-stimulated and basal AC activity. Histamine stimulated basal AC activity in guinea pig hippocampal membranes by 100% (o) from 16.1 to 31.4 pmols/mg/min. with an EC_{50} of 15 µM (R^2 = 0.97). In the presence of 10 µM forskolin, histamine (•) stimulated activity by 14% from 115 to 134 pmols/mg/min. with an EC_{50} of 26 µM. Stimulation of basal activity by PAT (□) was too low (<20%) to derive an EC_{50} value (R^2 = 0.68). Data points are means of three measurements with an average SEM of 0.61 (in the absence of forskolin) and 2.2 pmols (in the presence of forskolin).

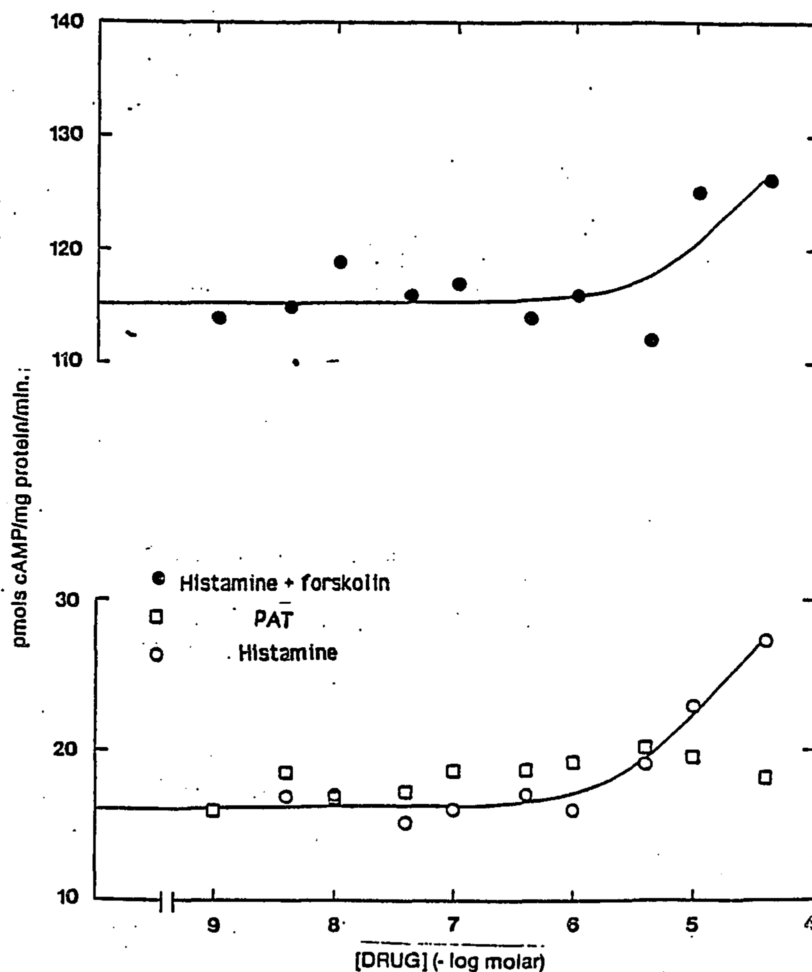


Figure 19 : Antagonism by spiperone, fluphenazine and mianserin of the stimulation by 5-HT of basal AC activity. 5-HT stimulated basal AC activity by 110% from 51.9 to 110 pmols/mg/min with an EC_{50} value of 94 nM. In the presence of 10 µM of each antagonist, 5-HT stimulated by 130% with an EC_{50} of 11 µM (spiperone, $R^2 = 0.99$), by 120% with an EC_{50} of 3.0 µM (fluphenazine, $R^2 = 0.996$) and by 120% with an EC_{50} of 1.1 µM (mianserin, $R^2 = 0.98$). Data points are means of three measurements with an average SEM of 1.6. Data were fit with equation 5 (no slope factor). Points preceding the breaks in the curve were measurements of basal activity in the absence of 5-HT and were included in the fit of the equation. In this experiment, 10 µM PAPP stimulated basal activity by 19%.

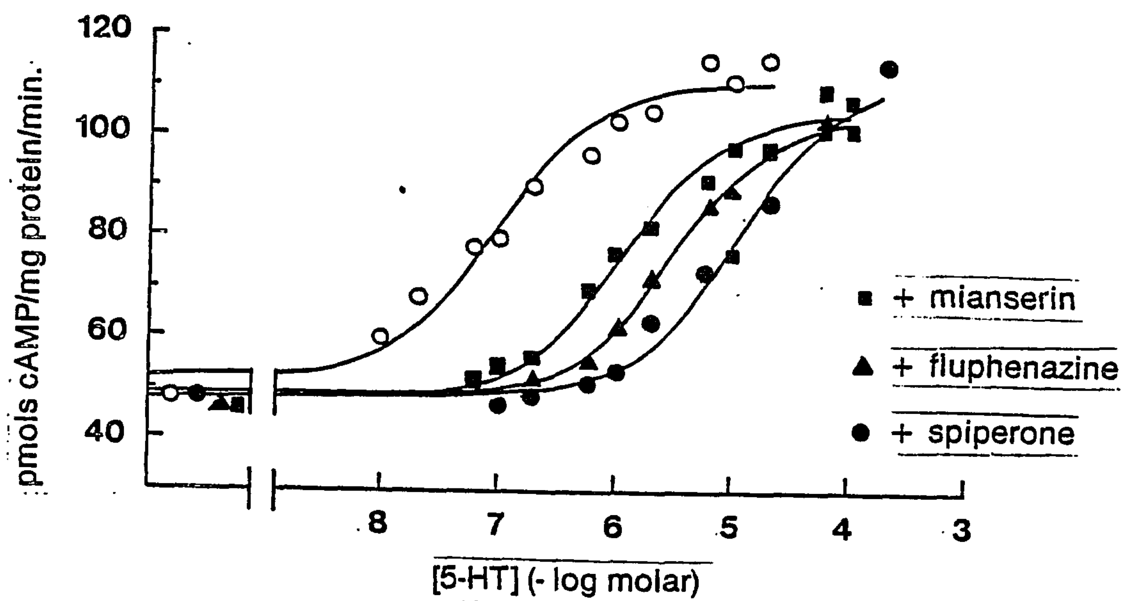
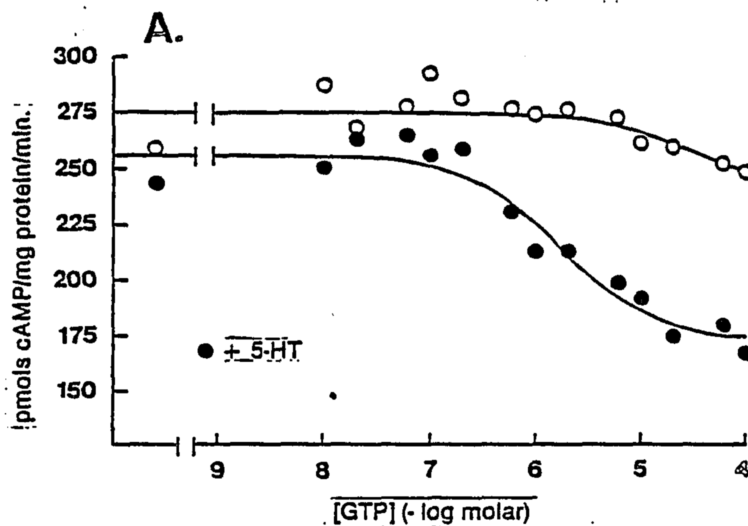


Figure 20 : Inhibition of adenylyl cyclase by GTP and Gpp(NH)p.

(A) GTP (•), in the presence of 10 µM 5-HT, inhibited AC activity by 32% from 256 to 173 pmols/mg/min (R^2 = 0.94) with an EC_{50} of 1.7 µM. In the absence of 5-HT (o), GTP produced, at most, a small (< 5%) decrease. (B) In the presence of 10 µM 5-HT, Gpp(NH)p (•) inhibited AC activity by 42% from 248 to 144 pmols/mg/min. (R^2 = 0.99) with an EC_{50} of 330 nM. In the absence of 5-HT, Gpp(NH)p (o) inhibited AC activity by 41% from 249 to 148 pmols/mg/min. (R^2 = 0.99) with an EC_{50} of 510 nM. Data points are the means of three measurements and the average SEM was 3.1 pmols/mg/min.



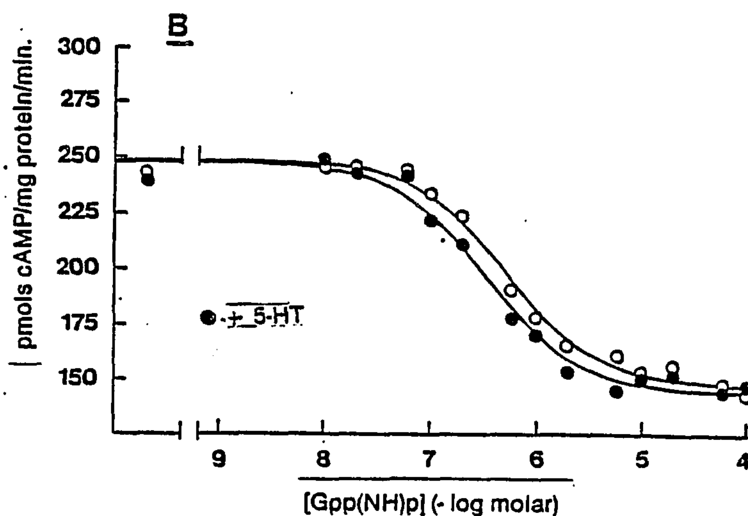


Figure 21 : Stimulation of adenylyl cyclase by GTP.
(A) GTP (o) stimulated basal AC activity from 12.5 to 32.7 pmols/mg/min. with an EC_{50} value of 1.1 µM. In the presence of 5-HT (•), GTP stimulated activity from 13.9 to 68.1 pmols/mg/min. with an EC_{50} value of 1.2 µM. Data points are the means of three measurements. R^2 values were 0.99 for both curves. (B) In the presence of 100 µM Gpp(NH)p, 5-HT (10 µM) stimulated AC activity from 345 to 454 pmols cAMP/mg protein/min. Data points are the means of three measurements.

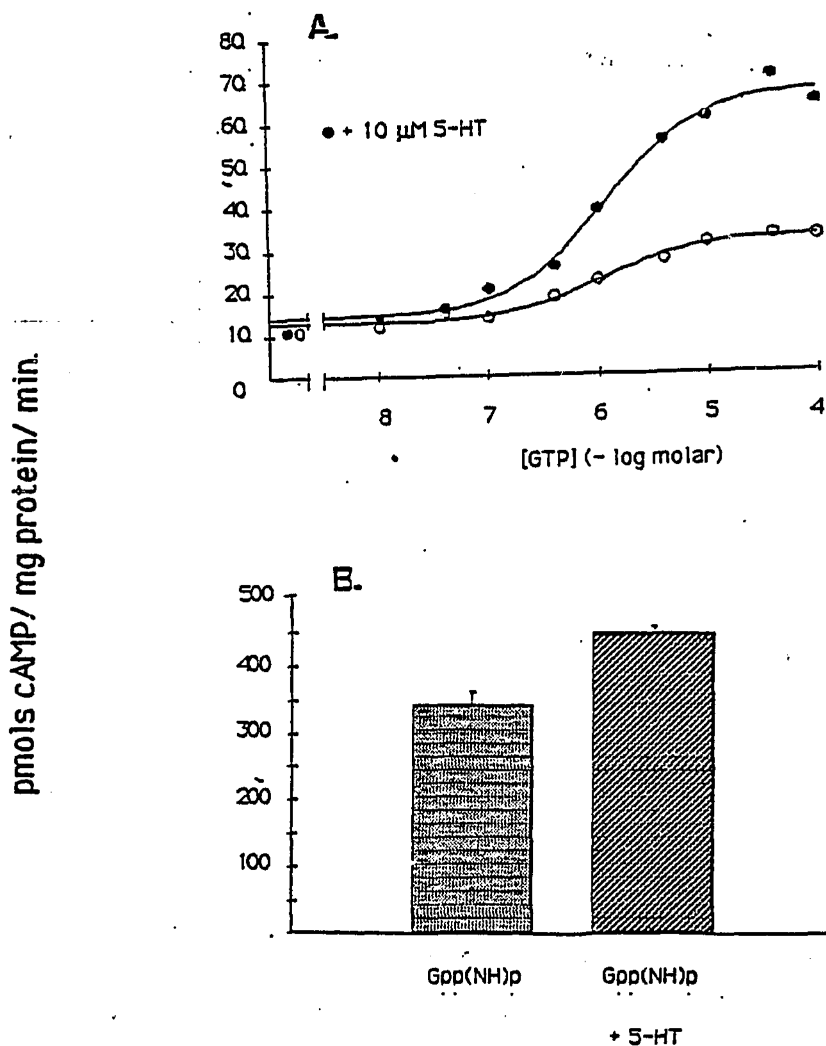


Figure 22 : Stimulation of basal adenylyl cyclase activity by Mg^{++}.

Mg^{++} stimulated basal AC activity from 11.7 to 362 pmols/mg/min. with an EC_{50} value of 6.2 mM. In the presence of 5-HT (•), Mg^{++} stimulated activcity from 15.6 to 419 pmols/mg/min. with an EC_{50} value of 2.8 mM. The R^2 values were 0.99 for both curves. Data points are the means of three measurements.

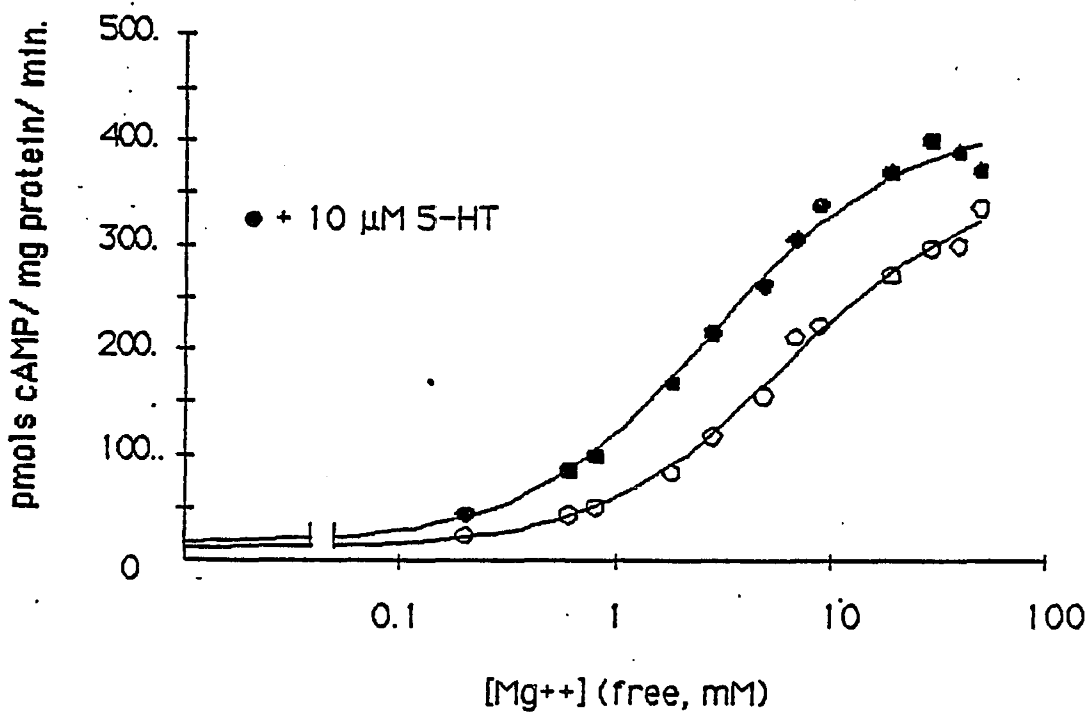


Figure 23 : Stimulation of adenylyl cyclase by Mg^{++} in the presence of forskolin. Mg^{++} (o) stimulated forskolin-stimulated AC activity from 44 to 410 pmols/mg/min. with an EC_{50} of 4.0 ± 0.4 mM (R^2 = 0.996). In the presence of 10 µM 5-HT, Mg^{++} (•) stimulated FSK-stimulated activity from 32 to 370 pmols/mg/min. with an EC_{50} of 4.0 ± 0.1 mM (R^2 = 0.997).

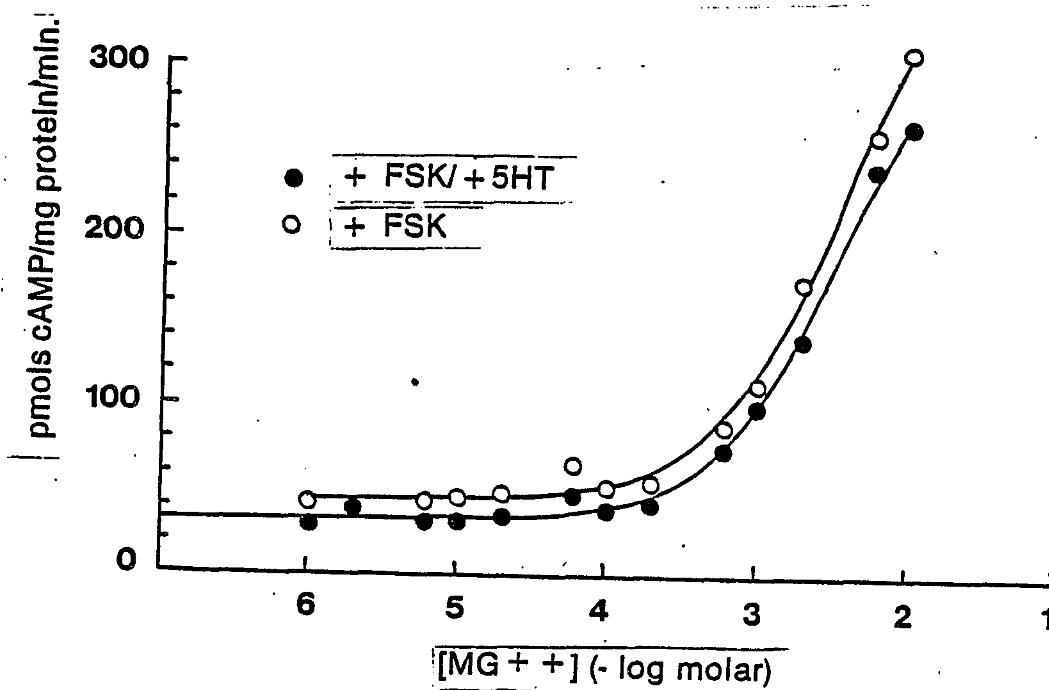


Figure 24 : Inhibition of adenylyl cyclase by 5-HT after reserpine treatment of guinea pigs. Data were normalized to 100. In guinea pig hippocampal membranes from a vehicle-injected guinea pig, 5-HT (o) inhibited AC activity by 26% from 315 to 233 pmols/mg/min. with an EC_{50} value of 45 nM (R^2 = 0.96). In membranes from a paired, reserpine-injected animal, 5-HT (•) inhibited activity by 27% from 241 to 175 pmols/mg/min. with an EC_{50} value of 16 nM (R^2 = 0.97). Data points are means of three measurements with an average SEM of 5.2. Points preceding the break in the curve represent activity in the absence of 5-HT and were included in the fit of the equation to the data.

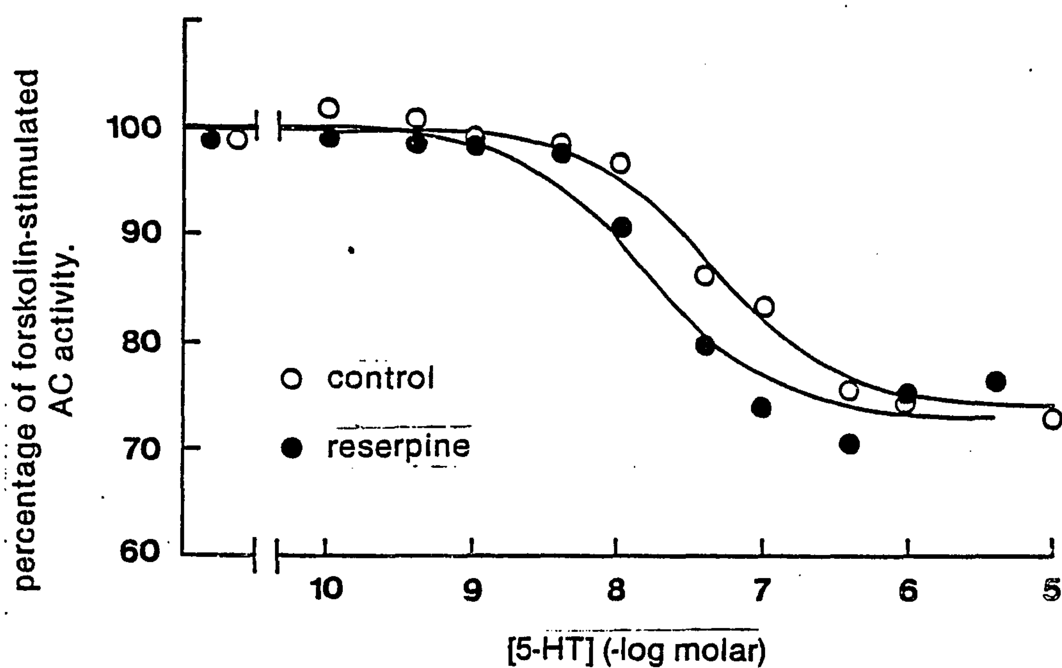


Figure 25 : Stimulation of basal adenylyl cyclase activity by 5-HT after reserpine or 5-HTP treatment of guinea pigs. Data from three different experiments were normalized to 100. In hippocampal membranes from a reserpine-treated guinea pig (Δ), 5-HT stimulated adenylyl cyclase activity by 224% from 58 to 189 pmols/mg/min. with an EC_{50} of 130 nM. In homogenates from a 5-HTP-treated animal (•), 5-HT stimulated activity by 59% from 120 to 191 pmols/mg/min. with an EC_{50} of 130 nM. A control curve to 5-HT is included for comparison (o). Data points are means of three measurements. The points preceding the break in the curve represent activity measured in the absence of agonist and were included in the fit of the equation to the data.

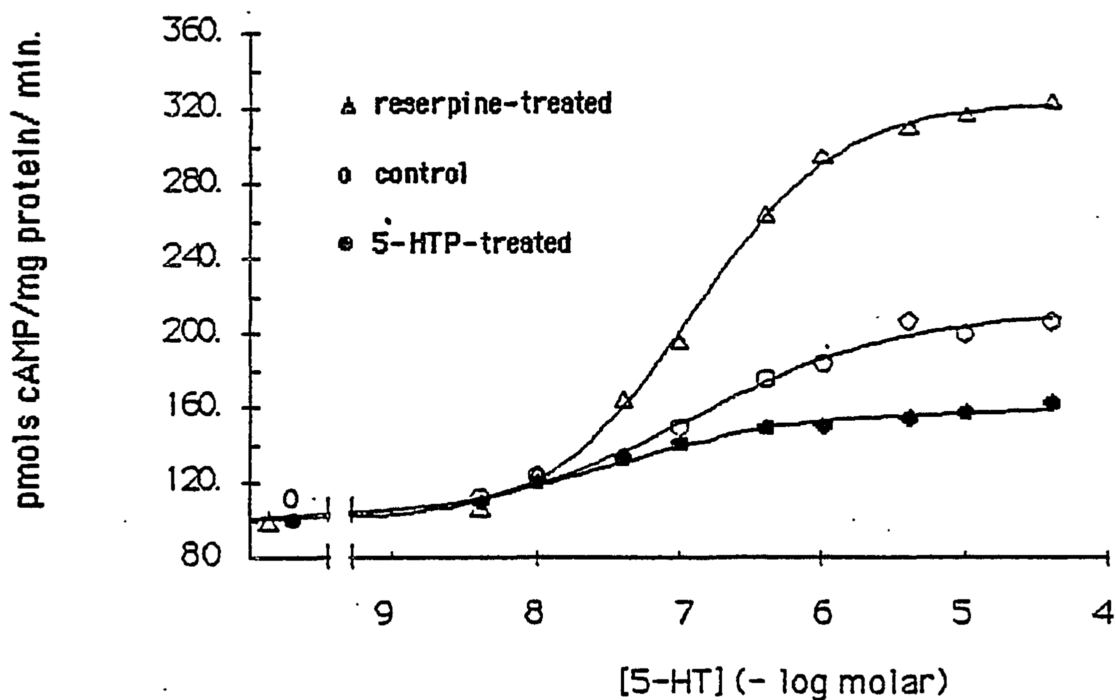


Figure 26 : Time course of the decrease in basal, 5-HT and histamine-stimulated AC activity in guinea pig hippocampal homogenates. The decrease in basal (□) activity with time is described by equation 11. AC activity decreased from 120 pmols/mg/min. and the first order decay constant was 0.02 min^{-1}. Histamine-stimulated (Δ) activity was a constant 2.9-fold stimulation of basal activity throughout the 60 min of the experiment. Similarly, incubation of the homogenate without agonist for the indicated period of time and exposure of the homogenate to 10 µM 5-HT (o) during the assay resulted in a constant 1.8-fold stimulation of basal activity. Homogenates incubated from t=0 with 10 µM 5-HT, however, experienced an additional decrease in activity with a rate constant of 0.26 min^{-1} (equation 18). All four sets of data were fit simulataneously for the estimate of the basal rate of decay.

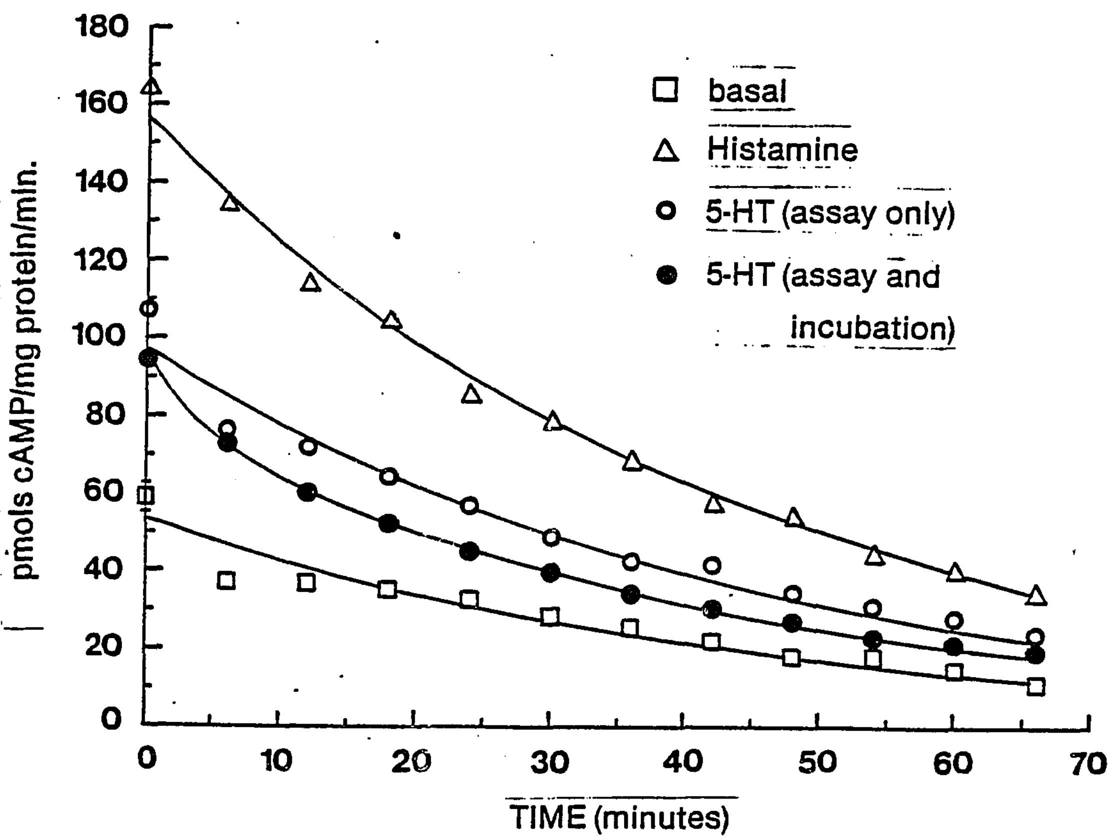


Figure 27 : Concentration-dependence of the 5-HT-mediated decrease in 5-HT-stimulated AC activity. Guinea pig hippocampal honomogenates were incubated with the indicated concentration of 5-HT for 30 min. At the end ot the incubation, the assay was started by the addition of [α-^{32}P]ATP and a concentration of 5-HT that elicits a maximal stimulation of AC activity (10 μM). Incubation of the homogenates with increasing concentrations of 5-HT reduced the subsequent maximal response to 5-HT. The maximal response to 5-HT during the assay was reduced by 5-HT during the incubation by 39% from 199 to 121 pmols/mg/min. with an EC_{50} of 50 nM.

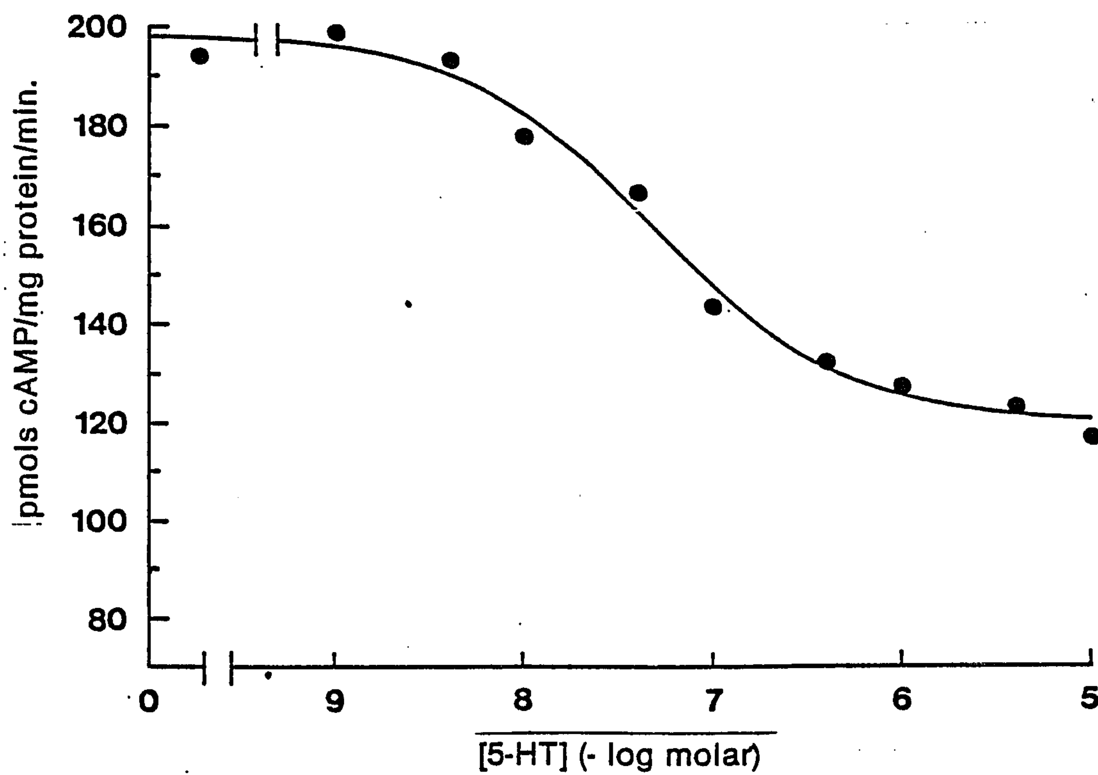


Figure 28 : Lack of "desensitization" to 5-HT after incubation of the homogenate in the absence of GTP and Mg++ and additivity of desensitization with the 5-HTP-mediated decrease in stimulation of AC by 5-HT. (A) In the absence of GTP or Mg++, incubation of the guinea pig hippocampal homogenates with 10 µM 5-HT does not reduce the responsiveness of the homogenate to 5-HT compared to homogenates incubated without 5-HT. In the control homogenates, incubation for 30 min with 10 µM 5-HT reduced the percentage of stimulation by 5-HT from 99% to 49%. (B) In a hippocampal homogenate from a 5-HTP-treated guinea pig, incubation of the tissue for 30 min with 10 µM 5-HT reduced the stimulation by 5-HT from 65 to 30%.

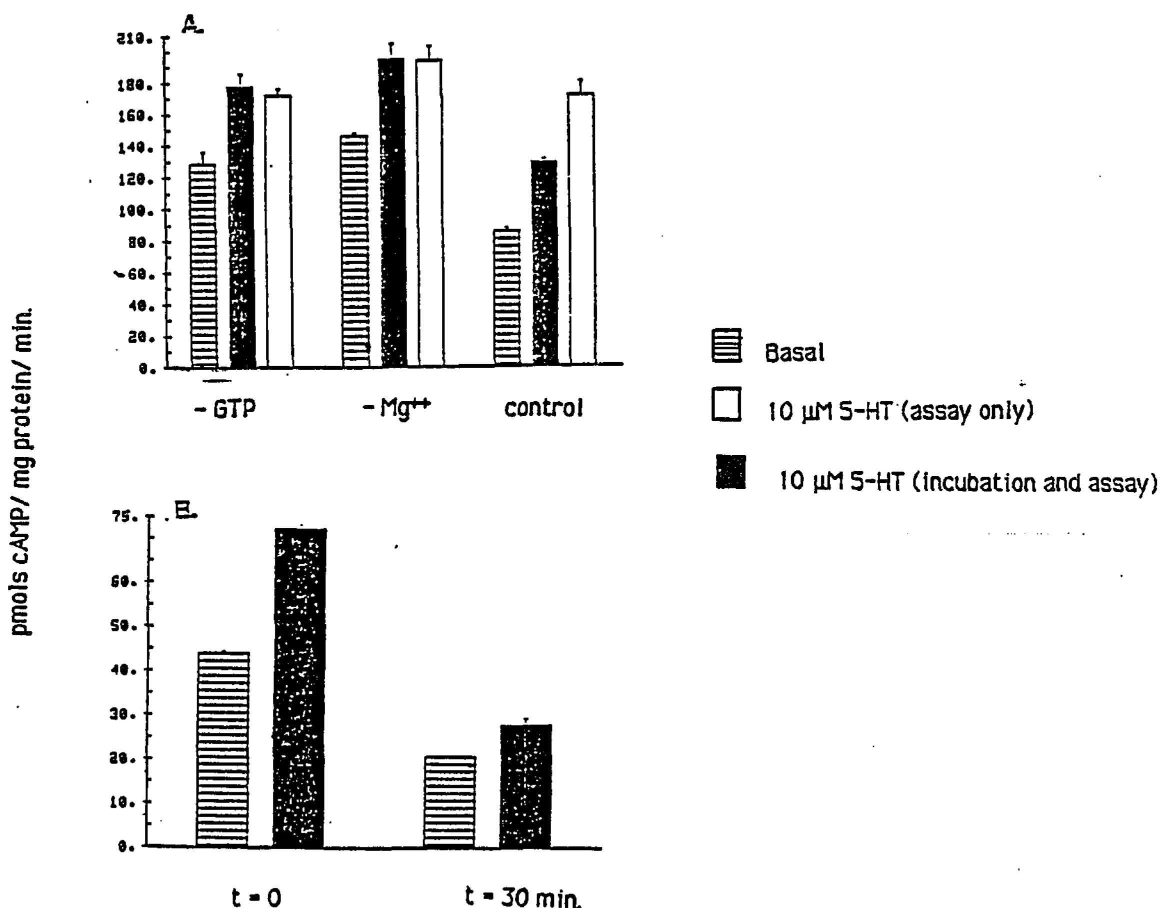


Figure 29 : Incubation of membranes with pertussis toxin reduces the inhibition by 5-HT. Incubation of guinea pig hippocampal membranes with 10 µg of pertussis toxin for 10 min reduced the percentage of inhibition by 10 µM 5-HT of forskolin-stimulated AC activity from 22 to 16% compared to membranes incubated with buffer. EC_{50} values of 5-HT were 52 nM (control) and 23 nM (pertussis toxin-treated).

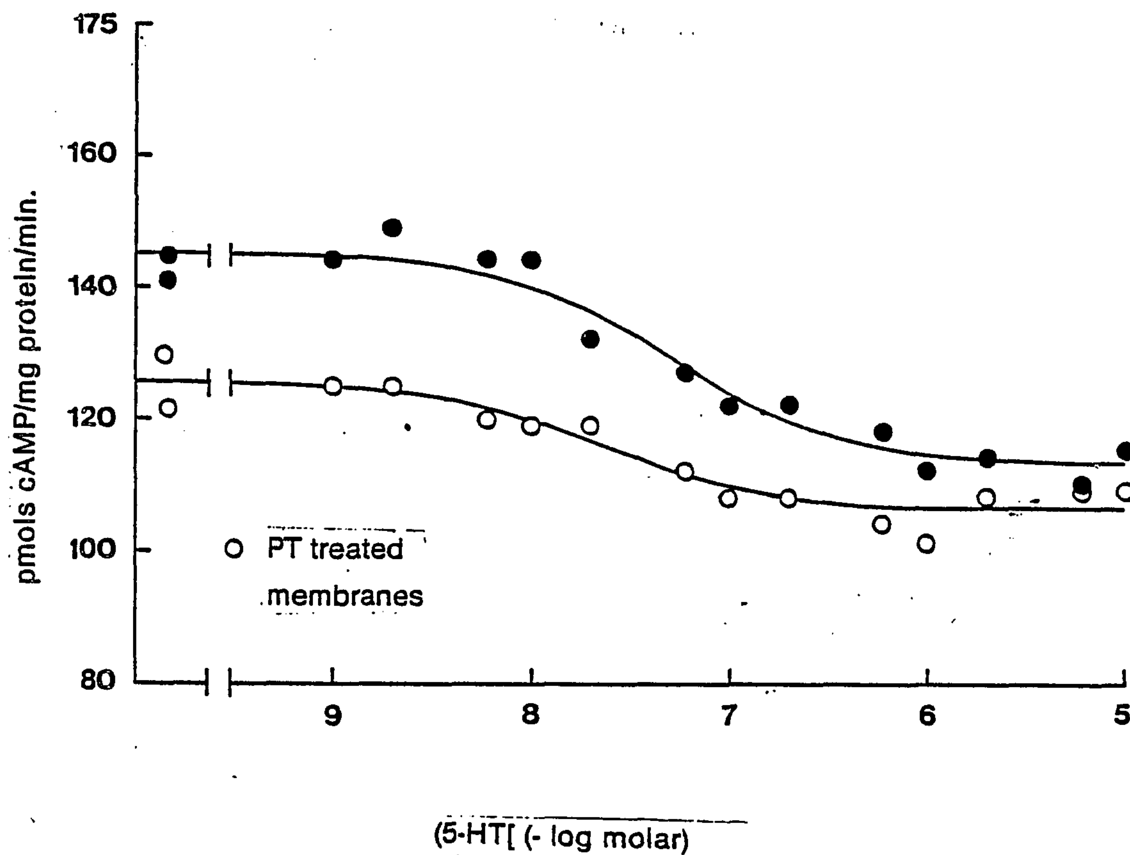
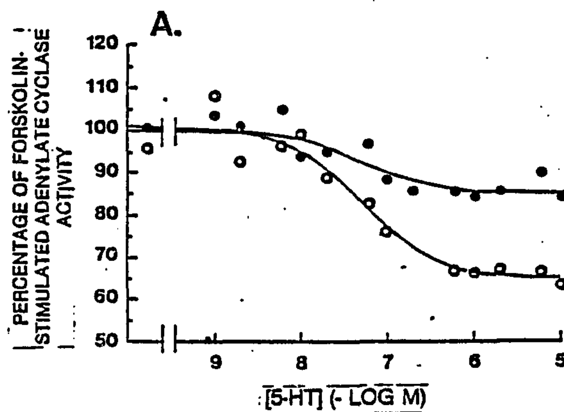


Figure 30 : Effect of pertussis toxin administered in vivo to rats on the inhibition of AC by 5-HT and the decrease in population spike amplitude by 5-HT. In two rats, administration of different doses of pertussis toxin resulted in corresponding decreases in 5-HT mediated effects on population spike amplitude (PSA) and forskolin-stimulated AC activity. (A) 5-HT (•), in a rat pretreated with 0.5 µg PT, inhibited AC activity by 32% from 109 to 71 pmols/mg/min. with an EC_{50} of 53 nM. In a rat treated with 1.5 µg of PT, 5-HT inhibited activity by 16% from 74 to 62 pmols/mg/min. with an EC_{50} of 46 nM. (B) In experiments done by William Clarke, 5-HT (10 µM) decreased the PSA by 61% in the rat treated with 0.5 µg. In the rat treated with 1.5 µg, 5-HT decreased the PSA by 9%. (For further explanation of the population spike amplitude data, see Clarke et al., 1987).



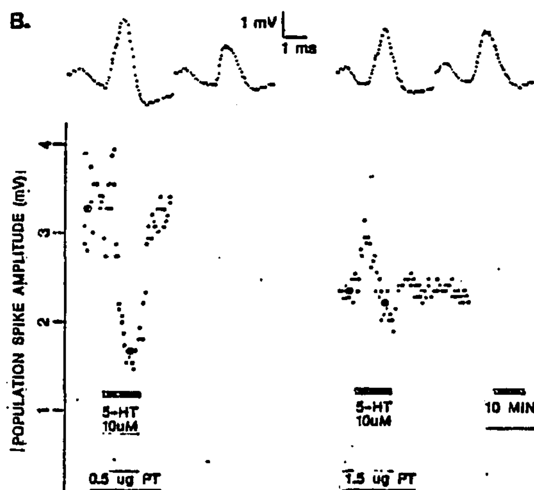


Figure 31 : Effect of a high dose of pertussis toxin on the inhibition of AC by 5-HT. In a rat treated with 3 μg of pertussis toxin, 5-HT (•) produced no observable inhibition of forskolin-stimulated adenylyl cyclase activity compared to a paired control animal that received a n injection of the vehicle alone.

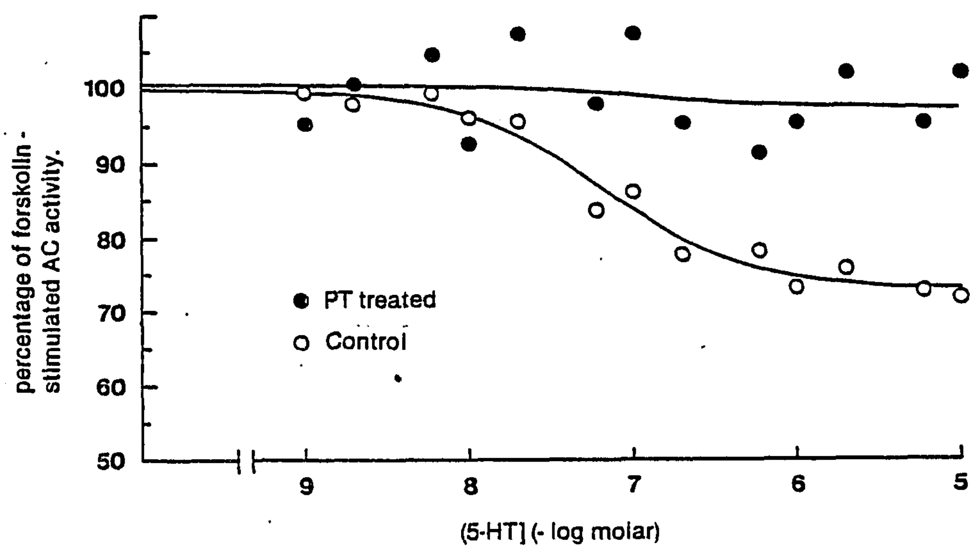


Figure 32 : Concentration-dependence of the pertussis toxin effect. (A) The maximal percentage of inhibition of AC activity by 5-HT was calculated by performing a complete concentration-response curve (as shown in fig. 31). Increasing doses of PT reduced the maximal percentage of inhibition by 5-HT of forskolin-stimulated AC activity. (B) Increasing doses of PT reduced the percentage of inhibition of the PSA by 10 µM 5-HT. The percentages from (A) and (B) were transformed by the arcsine transformation (Zar, 1984) because percentages are not normally distributed. The regression of the percentages in (A) with the dose of PT used was significant by the F test, $p = 0.0005$. The regression of the percentages in (B) with dose of PT was significant by the F test with $p = 0.034$. Data points are means of the indicated number of experiments; error bars are standard deviations of the means.

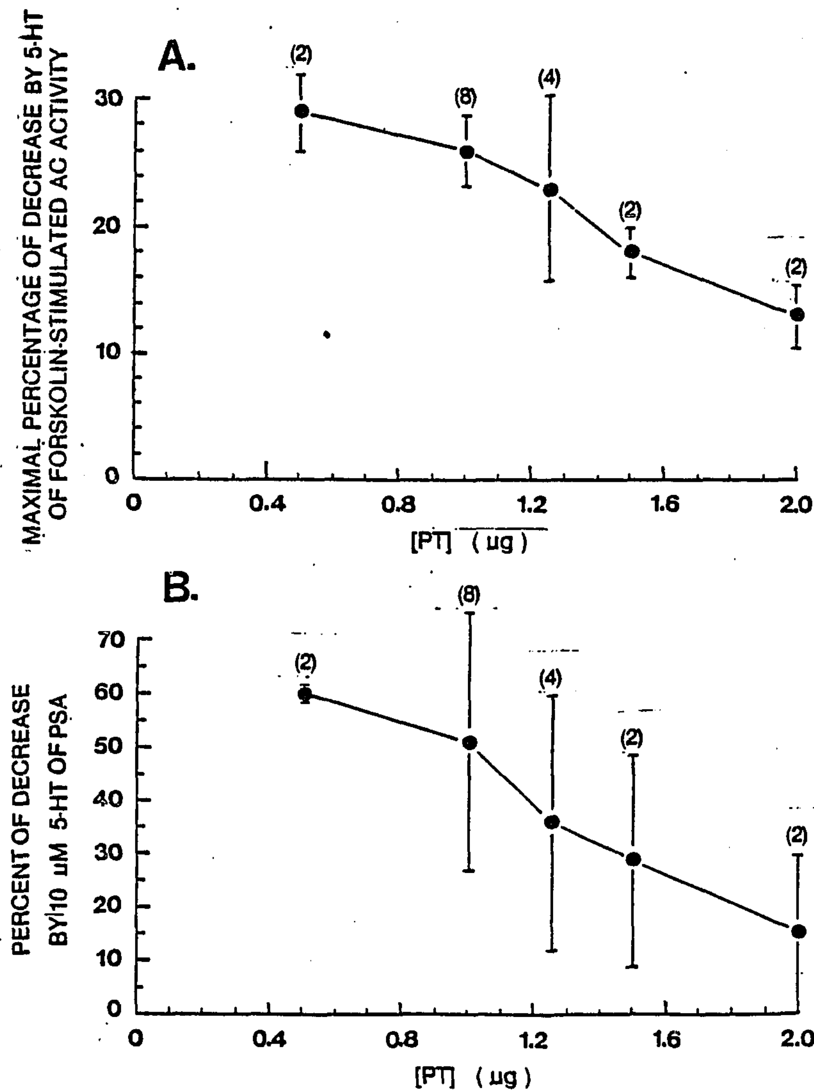
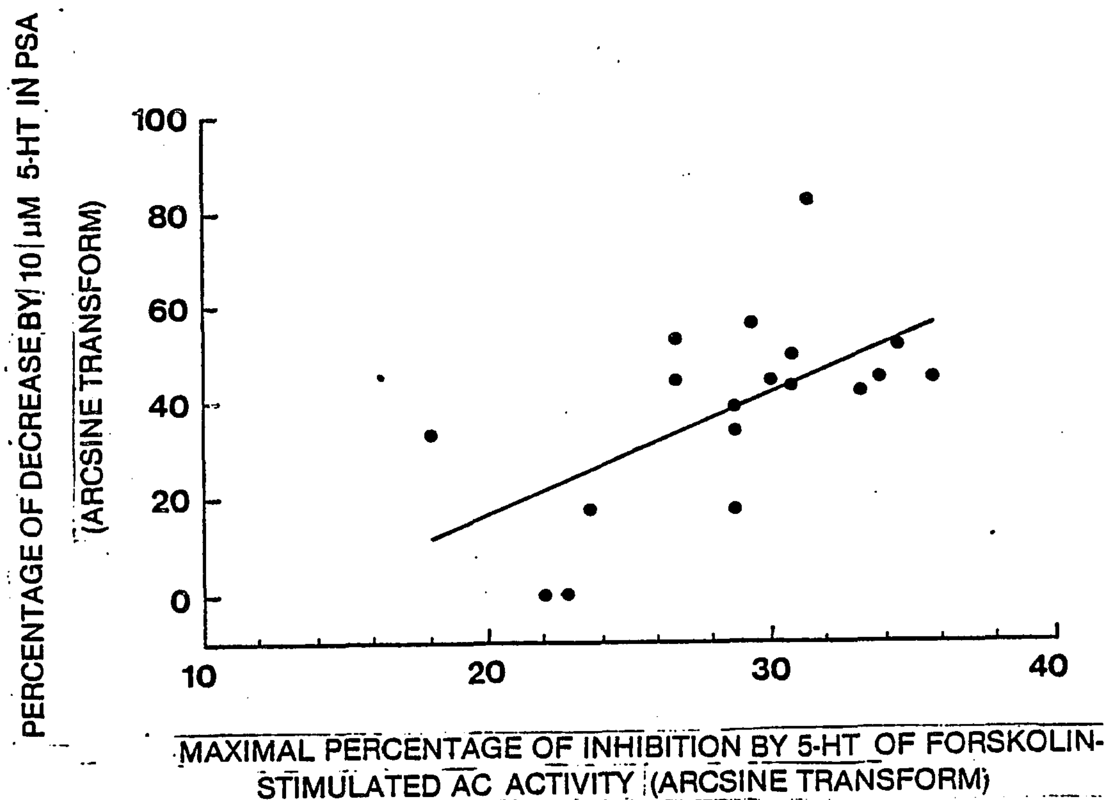


Figure 33 : Correlation of the magnitudes of the inhibition of AC and decrease in PSA by 5-HT. Percentages were transformed as described in fig. 32. The correlation of the maximal percentage of inhibition by 5-HT of forskolin-stimulated AC activity in rat hippocampal membranes with the percentage of decrease in population spike amplitude by 10 μM in rat hippocampal slices was significant ($p = 0.01$). Each point represents an experiment measuring AC activity and PSA in the same rat.



VI. Table of Results

Drug	Guinea pig		Rat	
	EC_{50}[a] (nM)	Inhibition[b] (%)	EC_{50} (nM)	Inhibition (%)
lisuride	N.D.[c]	N.D.	5.2 ± 0.2 (3)	27 ± 3
5-CT	5.1 ± 0.2 (7)[d]	28 ± 2	8.1 ± 0.4 (4)	30 ± 3
PAT	18 ± 1 (3)	29 ± 2	24 ± 3 (3)	23 ± 1
d-LSD	24 ± 3 (3)	39 ± 2	78 ± 5 (3)	27 ± 6
PAPP	27 ± 3 (3)	33 ± 3	N.D.	N.D.
5-MT	49 ± 5 (3)	27 ± 8	N.D.	N.D.
5-HT	53 ± 4 (7)	31 ± 4	109 ± 6 (7)	30 ± 4
RU 24969	59 ± 5 (3)	35 ± 9	N.D.	N.D.
Buspirone	146 ± 6 (3)	26 ± 6	170 ± 44 (3)	15 ± 5
Metergoline	153 ± 26 (3)	30 ± 4	N.D.	N.D.
Methysergide	260 ± 37 (3)	34 ± 7	N.D.	N.D.
TFMPP	900 ± 160 (3)	25 ± 5	N.D.	N.D.

[a] Values are geometric means ± S.E.M.

[b] Values are means ± S.D.s.

[c] Value was not determined.

[d] Numbers in parentheses are the number of experiments used to determine the parameter values.

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