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THE UNIVERSITY OF ALBERTA

INVESTIGATION OF ADENOSINE RECEPTORS IN RABBIT SMALL INTESTINE BY RADIOLIGAND BINDING

BY

RAVINDRA MUNSHI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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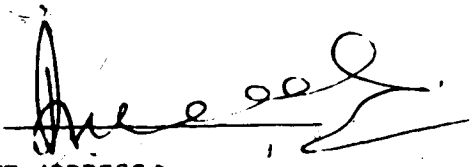
(SIGNED) 

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Investigation of adenosine receptors in rabbit small intestine by radioligand binding" submitted by Ravindra Munshi in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

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ABSTRACT

Adenosine and its derivatives decrease the spontaneous activity of isolated small intestine from rabbit. The role of cyclic AMP as a mediator of this relaxant effect is controversial. The rank order of potency of relaxants is not correlated with that for agonists at the A_2 receptor that stimulates adenylate cyclase to produce cAMP, nor is this stimulation demonstrable in membrane preparations from intestinal muscles. It has now been recognized that the adenosine receptors are diverse and may not be linked to adenylate cyclase in all cell systems.

To further investigate the receptor in intestinal smooth muscle, radioligand binding studies were undertaken using longitudinal-muscle membranes and a newly developed ligand of high specific radioactivity, [^{125}I]-N^6-R-(3-iodo,4-hydroxyphenyl)isopropyladenosine ([^{125}I]R-IHPIA). Initial characterization of the radioligand receptor interactions using adenosine receptors from rat brain revealed that the binding of [^{125}I]R-IHPIA was saturable and specific, and satisfied most criteria for radioligand binding to adenosine receptors.

However, [^{125}I]R-IHPIA binding to rabbit longitudinal-muscle membranes revealed features not consistent with binding to functional adenosine receptors. It exhibited a high nonspecific-component and could be displaced by compounds that are not known to have an affinity for the receptors. Use of another radioligand, [^{3}H]-N^6-R-(2-phenyl)-isopropyladenosine revealed similar characteristics of binding. The problem of anomalous specificity in ligand-site interactions was also evident when [^{3}H]-labelled 5'-N-ethylcarboxamidoadenosine (NECA) was

used to investigate the longitudinal-muscle membrane receptors. The specific binding of all three radioligands was not displaced by 8-p-sulphophenyltheophylline (SPT), which antagonizes the relaxant action of these compounds on the isolated intestinal tissue. Studies of dissociation of the bound radioligand at equilibrium revealed that these radioligands dissociated very rapidly thus not allowing measurement of the receptor specific binding either in a filtration or a centrifugation assay. These results show that there are difficulties with using these radioligands to characterize adenosine receptors in some systems.

5'-Deoxy-5'-methylthioadenosine (MTA) was found to be an agonist of the intestinal receptor and its relaxant effect was competitively antagonized by SPT. However, MTA competitively antagonized NECA-stimulated adenylate cyclase activity in murine neuroblastoma cell membranes. Our results with MTA provide strong evidence that the receptor in intestinal muscle is different from an A_2 receptor that stimulates adenylate cyclase.

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ABBREVIATIONS

AR:	Adenosine
CHA:	N^6-[Cyclohexyl]adenosine
PIA:	N^6-[2-(4-Phenyl)isopropyl]adenosine
HPIA:	N^6-[2-(4-Hydroxy)phenylisopropyl]adenosine
IHPIA:	N^6-[2-(3-Iodo,4-hydroxy)phenylisopropyl]adenosine
IABA:	N^6-(3-Iodo,4-aminophenyl)methyladenosine
IAPNEA:	N^6-[2-(3-Iodo,4-aminophenyl)ethyl]adenosine
NECA:	5'-N-Ithylcarboxamidoadenosine
NCPCA:	5'N-Cyclopropylcarboxamidoadenosine
MECA:	5-N-Methylcarboxamidoadenosine
CDA:	5'-Deoxy-5'-chloroadenosine
MTA:	5'-Deoxy-5'-methylthioadenosine
SPT:	8-(p-Sulfo)phenyltheophylline
SAH:	S-Adenosylhomocystein
HNBTG:	Hydroxynitrobenzylthionguanosine
NBMPR:	N^6-(4-nitro)benzylthioinosine
IBMX:	1-Isobutyl,3-methylxanthine
DPX:	1,3-diethyl,8-phenylxanthine
AMP:	Adenosine monophosphate
ADP:	Adenosine diphosphate
ATP:	Adenosine triphosphate
cAMP:	Cyclic AMP
GTP:	Guanosine triphosphate
GTPγS:	Guanosine-5'-(3-O-thio)triphosphate

Gpp(NH)p:	Guanyl-5'-(β,γ,imino)triphosphate
EHNA:	Erythrohydroxynonyladenine
dCF:	Deoxycoformycin
RO-20-1724:	4-(3-Butoxy-4-methoxy-benzyl)-2-imidazolidinone
DTT:	Dithiothreitol (Cleland's reagent)
EDTA:	Ethylenediaminetetracetic acid
BSA:	Bovine serum albumin
NEM:	N-Ethyl malefmide
PPO:	2,5-Diphenyloxazole
POPOP:	2,2'-p-Phenylenebis-[5-phenyloxazole]
PEI:	Polyethyleneimine

1. INTRODUCTION

1. INTRODUCTION

1.1 Overview

This dissertation reports attempts to investigate the receptor that mediates the relaxant action of adenosine on smooth muscle by means of radioligand binding. A radioligand with high specific radioactivity was developed with the hope that its use would provide further insight into the molecular mechanisms underlying adenosine-induced smooth muscle relaxation.

In the first part of the introduction the current knowledge of the pharmacological effects of adenosine is reviewed with special emphasis on smooth-muscle systems. The remainder of the introduction discusses the various radioligands that have been used in adenosine-receptor studies and the characteristics of their binding to adenosine receptors in various systems. A statement of the research objectives concludes this section.

1.2. Cellular metabolism of adenosine

The most obvious pathway for both the extracellular and intracellular formation of adenosine is the "ATP Pathway"-(ATP→ADP→AMP). The AMP thus formed is hydrolyzed to adenosine by endo-5'-nucleotidase intracellularly and by ecto-5'-nucleotidase extracellularly. The ATP pathway for adenosine formation appears to be operative in many cell systems during energy deficit (Arch and Newsholme, 1978). The source of extracellular adenosine is either from ATP (released as a co-transmitter along with acetylcholine or norepinephrine) or from

intracellular adenosine that crosses the cell membranes either passively or actively (c.f. Daly, 1980).

The second pathway for the formation of intracellular adenosine is the "Methylation Pathway." S-Adenosylhomocysteine (SAH), formed intracellularly by biological methylation reactions involving S-adenosylmethionine (SAM), is degraded by SAH-hydrolase to adenosine and homocysteine. This hydrolytic reaction is estimated to produce 14 to 23 mmoles of adenosine daily in adults and may be a major metabolic source of adenosine (Mudd and Poole, 1975).

A variety of routes for inactivation of adenosine by intracellular enzymes such as adenosine deaminase, adenosine kinase and SAH-hydrolase are present in cells. The function of these pathways appears to be the maintenance of low levels of endogenous adenosine. The first two inactivation pathways appear to operate together to influence the intracellular adenosine concentrations (see review by Daly, 1982). Further, in the presence of homocysteine, SAH-hydrolase can serve to trap adenosine as SAH, further modulating the intracellular adenosine levels.

1.2.1. Adenosine transport

Adenosine, as a hydrophilic molecule, would be expected to cross cell membranes only slowly by simple diffusion and indeed, it is now clear that most of the accumulation of adenosine in cells occurs by way of nucleoside-specific transport mechanisms (Plagemann and Wohlhueter, 1980; Paterson et al., 1981). The most extensively studied nucleoside transport system is the reversible, saturable,

non-concentrative, facilitated diffusion process which catalyses nucleoside fluxes that are many times larger than those attributable to simple diffusion. The driving force for this mode of nucleoside transport is the concentration gradient across the membrane. Although adenosine appears to be the preferred endogenous substrate for the transporter, the transport system has a fairly broad specificity allowing the transport of various physiological purine and pyrimidine nucleosides (cf. Paterson et al., 1985).

Various compounds are known to inhibit this nucleoside transport process and heterogeneity in nucleoside transport system with respect to different susceptibilities to inhibition by these comopunds has been reported. For example, a number of cultured cell lines such as KAB1 and KAB5 (both mutant clones from lymphoma S49) and Walker 256 rat carcinosarcoma and Novikoff rat hepatoma cells possess nucleoside transport systems that are either not inhibited or inhibited only by high concentrations of the nucleoside transport inhibitor N^6-(4-nitro)benzylthioinosine (NBMPR) (Belt, 1983; Aronow et al., 1985). Furthermore, both NBMPR-sensitive and insensitive transport systems have been reported to be present in L1210 mouse leukemia cells and HeLa cells (Dahlig-Harley et al., 1981; Belt, 1983). Species differences in the inhibition sensitivity of nucleoside transport systems have also been reported. Cardiac and CNS membranes prepared from rat tissues have a form of NBMPR binding site that has a much lower affinity for dipyridamole than sites in the guinea-pig tissues (Hammond and Clanachan, 1984; Williams et al., 1984).

In addition, a different form of nucleoside transport has also

been reported in renal and intestinal brush border vesicles. This is an active, sodium gradient-dependent, concentrative transport system which is not inhibited by NBMPR (S.M. Jarvis, personal communication; Le Hir and Dubach, 1984).

Vasodilation by the nucleoside transport inhibitor, dipyridamole, has been linked repeatedly to the ability of this compound to inhibit transport of adenosine (Olsson et al., 1972). An important determinant of biological activity of many other compounds such as NBMPR and related 6-thiopurine ribonucleosides, hexobendine, lidoflazine and dilazep is the susceptibility of nucleoside transport mechanisms to inhibition by these compounds. Nucleoside transport inhibitors have been shown, for example, to protect various neoplastic cells against the antiproliferative effects of a variety of cytotoxic nucleosides (Paterson, 1979). These inhibitors also potentiate the effects of adenosine in a wide variety of tissues. Dipyridamole, which increases coronary blood flow, (Feldman et al., 1981) also potentiates adenosine-induced increases in coronary blood flow. Similar effects have been observed with dilazep, its structural analog hexobendine (Marshall and Parratt, 1974; Fujii et al., 1981), and lidoflazine (Schaper et al., 1966; Van Neuten and Vanhoutte, 1980; Van Belle, 1970). Dipyridamole (Stafford, 1966; Hopkins, 1973), hexobendine, dilazep (Hopkins, 1973; Fujita et al., 1980), lidoflazine (Kucokhuseyin and Kaysalp, 1974) and diazepam (Clanachan and Marshall, 1980) have been shown to potentiate adenosine-induced negative chronotropic and/or inotropic effects.

Many other pharmacologically active compounds may produce at

least some of their effects through inhibition of nucleoside transport. Predominant among these are papaverine (Mustafa, 1979; Wu and Phillis, 1982), phenothiazines (Phillis and Wu, 1981a) and benzodiazepines (Phillis et al., 1981). The last group of compounds has been extensively studied with respect to their influence on adenosine accumulation in cells (Moritoki et al., 1985).

Adenosine derivatives such as cyclohexyladenosine (CHA) and phenylisopropyladenosine (PIA), which are highly lipophilic compounds, are not significantly transported by the carrier. These compounds, however, inhibit uridine influx in Novikoff rat hepatoma cells in a noncompetitive manner and in direct relation to their lipid solubility (Plagemann and Wohlhueter, 1984). This is a nonspecific effect and is not carrier mediated. On the other hand, the adenosine receptor agonist, 2-chloroadenosine, is a permeant for the nucleoside transporter in human erythrocytes (Jarvis et al., 1985) and guinea-pig cardiomyocytes (A.S. Clanachan, personal communication) and in each case, the transport of 2-chloroadenosine is inhibited by NBMPR in a competitive manner.

1.3 Pharmacology of adenosine and its derivatives

The pharmacological effects of adenosine were first described by Drury and Szent-Gyorgi (1929) in isolated frog hearts. Three decades later, Berne (1963) suggested a physiological role for adenosine in the control of coronary flow. Adenosine is now recognized as a ubiquitous hormone with no cell system uniquely responsible for its formation.

Although adenosine effects may result from altered purine metabolism (Arch and Newsholme, 1978; Fox and Kelly, 1978), some effects persist or are enhanced under conditions where the nucleoside is prevented from entering cells, using nucleoside transport inhibitors such as dipyridamole and NBMPR. Moreover, conjugates of adenosine with large molecules that are thought not to enter cells mimic the actions of free adenosine (Olsson et al., 1977; Fain and Malbon, 1979). Such findings suggested that cells contain surface receptors for adenosine.

Extracellular adenosine receptors have now been identified. This became possible when a variety of adenosine derivatives were developed agonists, and alkylxanthines were recognized as antagonists at adenosine receptors. In the following section, various agonists and antagonists at adenosine receptors will be reviewed together with the cAMP generating system that has been implicated in adenosine receptor-mediated biological responses, and a classification of adenosine receptors based on structure activity studies will be presented. This will be followed by a discussion of the physiological effects of adenosine.

1.3.1 Agonists of adenosine receptors

The adenosine molecule comprises 2 moieties, purine and ribose. Although numerous derivatives have been synthesized by various substitutions at C or N atoms, only a concise list of purine- and ribose-modified derivatives will be presented here based mainly on the utility of these compounds in adenosine receptor studies. Among the

purine modified derivatives are 2-chloroadenosine and various N^6-substituted compounds such as N^6-methyladenosine, N^6-phenyladenosine, N^6-benzyladenosine, N^6-(2-phenyl)isopropyladenosine (PIA, R- and S-diastereomers), N^6-[2-(4-hydoxy)phenylisopropyl]adenosine (HPIA, R- and S-diastereomers) and N^6-cyclohexyladenosine (CHA). Among the ribose-modified derivatives are: 2'-, 3'-, 5'-, and 2',5'-deoxyadenosine. The ribose-modified 5'-derivatives such as 5'-N-ethylcarboxamidoadenosine (NECA) and 5'-N-cyclopropylcarboxamidoadenosine (NCPCA) have proved extremely useful in adenosine receptor studies (Appendix I; see review by Bruns, 1980; Daly, 1982).

1.3.2 Antagonists of adenosine receptor

Alkylxanthines such as theophylline and caffeine were the first known antagonists at adenosine receptors (Fredholm, 1980). Although these compounds also inhibit the enzyme, phosphodiesterase (Berthet et al., 1957), and mobilize Ca^{2+} ions (Johnson and Inesi, 1969), the latter effects are seen only at concentrations about 100-fold higher than that required for the receptor antagonism. Thus, theophylline and caffeine probably owe many of their pharmacological effects to blockade of adenosine-mediated functions. Both theophylline and caffeine are relatively weak adenosine antagonists (affinity constants 10 - 50 μM, see Daly, 1982). Other substituted alkylxanthines with higher affinity have been synthesized. These are 1,3 diethyl-8-phenylxanthine (DPX), 8-phenyltheophylline, and its water-soluble analog 8-(p-sulpho)phenyltheophylline (SPT) which is now used extensively as an antagonist for adenosine receptors (Daly et al.,

1985). It has been suggested that SPT acts selectively on extracellular adenosine receptors, and does not inhibit phosphodiesterase, because owing to its charge in aqueous solution it penetrates cells to a negligible extent. As yet, no selective xanthine antagonists for adenosine receptor subtypes have been developed (see Bruns, 1981), although 1,3-dipropyl-8-(2-amino-4- chlorophenyl)xanthine (PACPX) is 70,000-fold more potent than theophylline in bovine brain (Bruns et al., 1983), and is at least 1,000 fold more potent than theophylline in rat fat cells, rat brain, and human platelets (Schwabe et al., 1985). Recently, the synthesis of a series of 8-phenyl-1,3,dialkyl xanthines has been reported. These compounds have high affinity (K_D<100 nM) at brain adenosine receptors, can be radioiodinated and may also be useful in the preparation of affinity columns (see Jacobson et al., 1985). Although adenosine-receptor agonists that are nucleosides have a strict requirement for the ribose moiety and must exist in a stable anticonfiguration, addition of a ribose moiety to theophylline at C_9 (theophylline 9-β-D-riboside) in a similar conformation to that in the adenosine molecule abolishes the antagonistic activity of theophylline (Clanachan, 1981).

Certain 5'-modified adenosine derivatives have been shown to be competitive antagonists at adenosine receptors (Burns, 1980). Predominant compounds among these are 5'-deoxy-5'-methylthioadenosine (MTA) (Appendix I) and 5'-deoxy-5'-carboxyadenosine methylester. Of these, MTA is an endogenous product of polyamine biosynthesis, generated from S-adenosylmethionine (Pegg and Williams-Ashman, 1969; 1970). MTA can also be formed directly from S-adenosylmethionine

(Swiatek et al., 1973; Wilson et al., 1979). Both xanthine and nucleoside antagonists have been used in the present study of smooth muscle adenosine receptors.

1.3.3 Cyclic AMP-generating systems

Adenylate cyclase is a ubiquitous enzyme that catalyses the conversion of ATP to cyclic AMP (cAMP) and PPi. Various hormones can either stimulate or inhibit the activity of this enzyme in various cell types (see review by Cooper, 1982), thereby effecting the transmission of messages through control of intracellular cAMP levels. The message-transmission system is composed of a hormone (H), its receptor (R), the regulatory proteins Ns and Ni, which bind the guanine nucleotide and the catalytic unit (C). Both Ns and Ni have inherent GTPase activity. The binding of hormone to its adenylate cyclase-associated receptor initiates the exchange of inactive GDP for active GTP at Ns or Ni. The activated Ns or Ni now activates C, which, in turn, utilizes the ATP - Mg^{2+}/Mn^{2+} complex to alter intracellular cAMP levels. GTP is quickly hydrolyzed to inactive GDP by GTPase and the system returns to the basal level. Both stimulation and inhibition of adenylate cyclase by hormones are GTP-dependent. In addition, Na^{+} (20 - 140 mM) has been shown to increase hormonal inhibition of adenylate cyclase (see review by Jakobs et al., 1981), although this is not true for every system. Sodium is thought to affect adenylate cyclase inhibition by reversing the GTP-induced inhibition of the activity and thus potentiating the hormone-induced inhibition (Cooper, 1982). Hormone-induced inhibition versus

stimulation of adenylate cyclase is greater at lower temperatures (20°C compared with 37°C), lower Mg^{2+} concentrations (1 - 2 mM compared with 5 mM or more), higher GTP concentrations (0.2 - 6 μM or more compared to 0.01 - 1 μM). Inhibition is also more sensitive to N-ethylmaleimide (NEM).

Adenylate cyclase can also be stimulated directly by GTP and its nonhydrolyzable derivatives such as 5'-guaninylimidodiphosphate (Gpp(NH)p) and guanosine-5'-(3-O-thio)triphosphate ($GTP\gamma S$) (Hildebrandt, 1983). However, low concentrations of $GTP\gamma S$ inhibit the enzyme's activity through Ni and this effect can be used to determine the presence of Ni in cells.

C can be directly activated by a plant diterpine, forskolin, in most cell systems (Seamon et al., 1981; Seamon and Daly, 1981a). Furthermore, forskolin enhances the ability of stimulatory as well as inhibitory hormones to affect enzyme activity. Although forskolin does not require GTP for its action (Seamon and Daly, 1981b), an interaction of forskolin with N proteins cannot be ruled out (Barovsky et al., 1984).

1.3.4 P site for Adenosine

Londos and Wolff (1977) [redacted] enosine inhibits adipocyte adenylate cyclase not only thr[redacted]ptors (see below) but also through the intracellular P site. [redacted] significance of the latter is unclear since adenosine concentrations in various types of cells are not thought to increase above 1 - 2 μM whereas the affinity of adenosine for this site is about 10-fold higher. The P site is

generally resistant to modifications in the purine ring (hence its name) with the result that 2-chloroadenosine has very low affinity (<2-fluoroadenosine) at this site and N^6-derivatives are totally inactive (see review by Daly, 1982). Some ribose-modified derivatives such as 2'-deoxyadenosine, 3'-deoxyadenosine, 5'-deoxyadenosine, 2',5'-dideoxyadenosine and adenine arabinoside are active at the P site, whereas others, for example, NECA, NCPCA, and MTA are inactive (Bruns, 1980). Adenine and inosine are also inactive at this site. Theophylline has no effect on the P site inhibition of adenylate cyclase (see review by Bruns, 1980).

1.3.5 Adenosine Receptors

It was observed for the first time in 1970 that adenosine increased the levels of cAMP in brain slice preparations (Sattin and Rall, 1970; Shimizu and Daly, 1970). A key to confirming the existence of adenosine receptors was the discovery that adenosine at low concentrations stimulated adenylate cyclase from a particulate fraction of platelets, whereas, at higher concentrations, it inhibited the enzyme (Haslam and Lynham, 1972). In similar experiments, 2-chloroadenosine was found to be at least as potent an activator of the enzyme as adenosine, but a weaker inhibitor. This suggested that the two actions of adenosine might have different structural determinants. Adenosine-mediated stimulation and inhibition of adenylate cyclase was subsequently reported for numerous types of cells. Two functionally and pharmacologically distinct extracellular adenosine receptors have now been identified as being associated with adenylate

cyclase (Van Calker et al., 1979; Londos et al., 1980), one mediating stimulation of its activity, the other inhibition. These are in addition to the intracellular P site for adenosine (Londos and Wolff, 1977).

1.3.5.1 Nomenclature of Adenosine Receptors

Several forms of nomenclature have been proposed for different sites of adenosine action. Burnstock (1978) suggested P_1 for adenosine receptors and P_2 for purine nucleotide receptors. Londos et al. (1980), proposed Ri and Ra for the adenosine receptors that mediate inhibition and activation of adenylate cyclase respectively. In 1979 Van Calker et al. proposed A_1 (inhibitory) and A_2 (stimulatory) for these same receptors. Since adenosine receptors in all cell systems may not be linked to adenylate cyclase, the terminology A_1 and A_2 will be used throughout this text to indicate receptors with high affinity (also inhibitory to adenylate cyclase) and low affinity (also stimulatory to adenylate cyclase), respectively (see Stone, 1984, 1985; Hamprecht and Van Calker, 1985).

1.3.5.2 Adenosine Receptor Subtypes

Although A_1 and A_2 adenosine receptors are undoubtedly discrete proteins, the first interacting with the Ni-subunit and the other with the Ns-subunit of adenylate cyclase, their recognition sites for agonists and antagonists show only quantitative differences. Thus, there are no truly specific agonists and most antagonists of the xanthine class are relatively nonselective.

xanthine class are relatively nonselective.

1.3.5.2.1 A_1 Receptor

Adenosine and most of its active derivatives have higher aff at this class of receptors than at the A_2 receptors. Amon various deriyatives, N^6-substituted compounds such as PIA and CH the most potent. This class of receptors is further characteriz at least a 10-fold difference in the affinities of R- S-diastereomers of PIA. 2-Chloroadenosine has the same affini both A_1 and A_2 receptors. The affinity of adenosine derivatives receptors is usually decreased by substitutions at 5'-positions. receptors have been demonstrated in purified fat-cell memb (Londos et al., 1978), in crude membrane pellets from brain c (Cooper et al., 1980), and in purified striatal and hippoc membranes (Yeung and Green, 1983a, 1984). The inhibitory aden receptor-adenylate cyclase system requires higher concentration GTP than the stimulatory action. The presence of Gpp(NH)p, concentrations of Mg^{2+} and low concentrations of Mn^{2+}, and h assay temperatures mask inhibition mediated by A_1 receptors (Coop al., 1980). Sodium ions in the range of 100 mM enhance A_1 rece mediated inhibition. Thus, in all respects, the inhibitio adenylate cyclase mediated by A_1 adenosine recept characteristics identical to those seen with other receptor sy inhibitory to adenylate cyclase from a variety of cells. Be adenosine is formed from ATP in the incubations, labelled deoxyA a preferred substrate; as well the presence of adenosine deam

(which inactivates adenosine) greatly facilitates the detection of inhibition through A_1 receptors (Cooper et al., 1980).

Recently, brain A_1 receptor has been photolabelled using various radioligands such as [^{125}I]-Azidobenzyladenosine (Choca et al., 1985) [^{125}I]-Aminophenylethyladenosine (Stiles et al., 1985) and [^{125}I]-2-Azido,N^6-p-hydroxyphenylisopropyladenosine (R-AHPIA) (Klotz et al., 1985). The reported molecular weight of A_1 receptor or a subunit of it is between 35,000-38,000 D.

1.3.5.2.2 A_2 Receptor

Among the various derivatives of adenosine, 5'-substituted compounds such as NECA and NCPCA are the most potent at A_2 receptors. N^6-substitutions decrease the affinity at A_2 receptors and the stereoselectivitiy of the diastereomers of PIA is only of the order of 3 - 5 fold. The presence of a group with relatively high lipophilicity at the 5'-position of adenosine derivatives (as, for example, in MTA) results in potent antagonist activity at A_2 (see review by Daly, 1985). A_2 receptors have been demonstrated in purified membranes from liver cells (Cooper and Londos, 1979; Schutz et al., 1982), human platelets (Huttermann et al., 1984), Leydig cell tumor (Londos et al., 1980), rat striatum (Yeung and Green, 1983, 1984), and in other tissues. Lower concentrations of GTP are required for A_2 receptor-mediated adenylate cyclase stimulation than for its inhibition through A_1 receptors. The presence of Gpp(NH)p, higher concentrations of Mg^{2+}, and higher temperatures facilitate stimulation

of the enzyme through A_2 receptors. Sodium ions do not affect A_2 stimulation of adenylate cyclase activity.

1.3.6 Central Effects

1.3.6.1 Behavioral Responses

Adenosine and several of its metabolically stable derivatives are potent modulators of central nervous system (CNS) function. Behaviorally, adenosine and its derivatives produce sedation, anticonvulsant effects (Maitre et al., 1974), anxiety (Barraco et al., 1984), hypothermia and antinociception (Vapaatalo et al., 1975), analgesia (Stone and Perkins, 1979), sleep and depression (Williams, 1983a), muscle relaxation and ataxia (Baird-Lambert et al., 1980; Buckle and Spence, 1981), depressed respiration (LagerCrantz et al., 1984), and inhibition of apomorphine-induced rotation behavior (Fredholm et al., 1983a). In general, these effects can be antagonized by alkyl xanthines such as caffeine and theophylline (which themselves are central stimulants) at doses 3 - 300 times lower than those causing phosphodiesterase inhibition and calcium mobilization (see reviews by Daly et al., 1981; Williams, 1984; Barraco, 1985). Besides methylxanthines, the action of other central stimulant and depressant agents such as ethanol, morphine, diphenylhydantoin, meprobamate, benzodiazepines, barbiturates and tricyclic agents (with sedative properties) may involve adenosine (Phillis, 1985).

1.3.6.2 Biochemical Responses (Modulation of Adenylate Cyclase)

Adenosine has been shown to stimulate cAMP production in brain slices from virtually all species and regions studied (Skolnick and Daly, 1977; Fredholm et al., 1982). At least two subclasses of such stimulatory adenosine receptors appear to be present in brain tissue (Daly et al., 1983): a ubiquitous low-affinity receptor (EC_{50}=10 - 20 μM) detected only in slices (from all brain regions) and not in assays of adenylate cyclase using brain membranes, and a relatively high-affinity receptor (EC_{50}=0.5 μM) detectable only in membranes from striatal and other limbic areas (Premont et al., 1979).

Decreases in the basal cAMP level in brain slices induced by adenosine derivatives were not demonstrable (Fredholm et al., 1982), although the same authors have shown a PIA-induced small decrease in forskolin-stimulated accumulation of cAMP in rat hippocampal slices (Fredholm et al., 1983b). Furthermore, A_1 receptor-mediated inhibition of adenylate cyclase activity is demonstrable in cell-free systems from most brain regions (Londos et al., 1980; Yeung and Green, 1984) and in cultured glial cells (Van Calker et al., 1979).

1.3.6.3 Electrophysiological Responses

At the electrophysiological level, adenosine causes hyperpolarization of central neurons concomitant with depression of both spontaneous and evoked neuronal activity (Phillis and Wu, 1981b; Stone, 1981). These findings have been generally attributed to a reduction in the release of excitatory synaptic transmitter (Phillis and Wu, 1981a), the nucleoside (adenosine) having been shown to

prevent the release of acetylcholine, dopamine, norepinephrine, gamma-amino butyric acid, glutamate and serotonin (see Williams, 1984).

In view of the available evidence that, if cAMP has any effect, it is to facilitate transmitter release (Drummond, 1983), adenosine-induced accumulation of cAMP in slices is not consonant with inhibition of transmitter release (Smellie et al., 1979; Dunwiddie and Hoffer, 1980). The potent cAMP derivative, 8-p-chlorophenylthio-cyclic AMP, neither mimicked nor antagonized the depressant effect of adenosine on synaptic transmission. Furthermore, heavy metals such as lead that are known to disrupt the hormonal regulation of adenylate cyclase (Taylor et al., 1978) have no significant effect on depressant responses to adenosine in hippocampus (Dunwiddie and Fredholm, 1985). Adenosine and isoproterenol have been reported to have marked synergistic effects on brain-slice cAMP levels (Cooper et al., 1980; Huang et al., 1971). However, isoproterenol has no effect on the magnitude of adenosine-mediated depressant responses in brain slices (Dunwiddie and Fredholm, 1985). Thus, adenosine-mediated cAMP accumulation can be either inhibited or stimulated without markedly affecting electrophysiological responses to adenosine (Dunwiddie and Fredholm, 1984). These findings suggest that adenosine may elicit some types of response through mechanisms other than changes in adenylate cyclase activity (see below).

1.3.7 Effects on Smooth Muscle

1.3.7.1 Excitation-Contraction Coupling in Smooth Muscle

All smooth muscle cells contain the contractile proteins actin, myosin, and tropomyosin (Stull, 1980). An increase in the intracellular Ca^{2+} concentration activates the calcium-binding protein, calmodulin (Cheung, 1980). The inactive myosin light-chain kinase (MLCK) is activated by the Ca^{2+} - calmodulin complex and subsequently phosphorylates the myosin light chain (MLC). In the presence of the contractile protein actin, phosphorylated-MLC activates the Mg^{2+} - ATPase activity of myosin. The subsequent cleavage of ATP yields energy which is utilized in muscle shortening according to the sliding filament theory (see Stull and Sanford, 1981; Rasmussen and Barret, 1984). The biochemical events that are thought to take place during smooth-muscle relaxation mediated by beta-adrenergic receptors are diagrammatically represented in Figure 1.

1.3.7.2 Possible Mechanisms of Smooth Muscle Relaxation

Sutherland and Rall (1960) first suggested that hormone induced relaxation of smooth muscle may result from an increase in intracellular levels of cAMP. Catecholamines, neurotransmitters and many peptide hormones increase cAMP formation in smooth-muscle tissues by stimulating specific membrane receptors (Kamm and Stull, 1985). The question of cAMP involvement in smooth-muscle relaxation mediated by beta adrenergic agonists has been extensively reviewed over the past decade (Baer, 1974; Anderson et al., 1975; Namm and Leader, 1976;

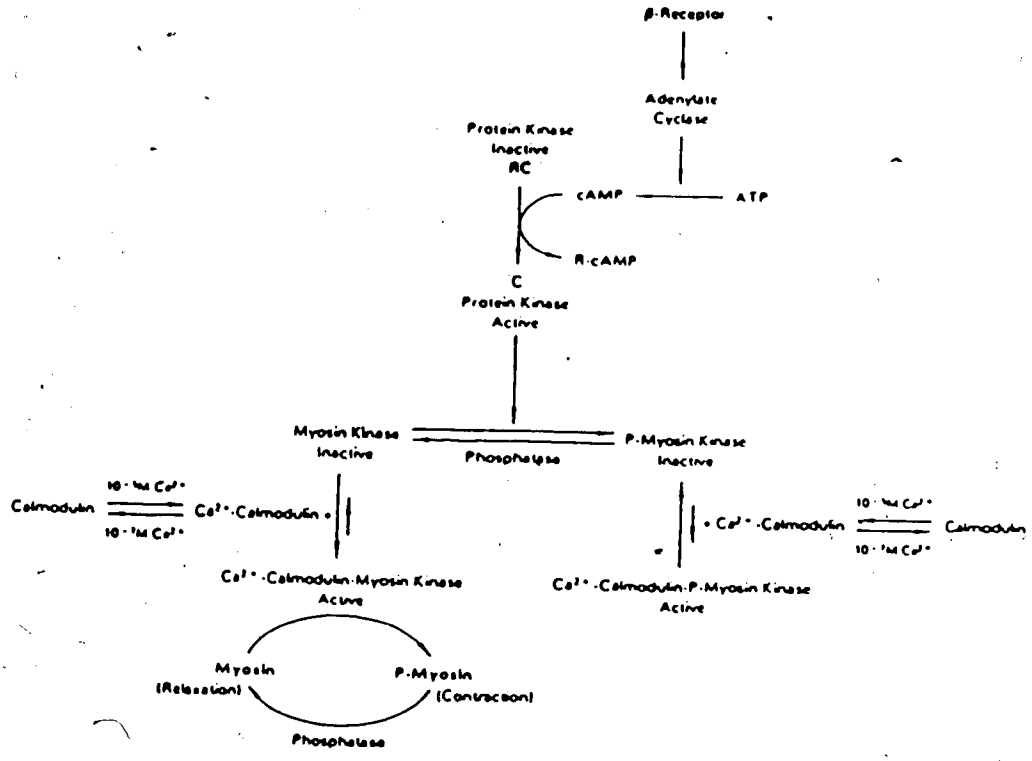


Fig. 1. Schematic representation of beta-adrenoceptor-stimulated adenylate cyclase activity and subsequent events leading to smooth muscle relaxation. This is a composite diagram of models proposed by Rodbell (1980), Adelstein et al. (1978) and Scheid et al. (1979).

Anderson and Nilsson, 1977; Hardman et al., 1977; Diamond, 1978; Kramer and Hardman, 1980; Adelstein et al., 1978; Kamm and Stull, 1985) and in general, a correlation has been found between elevated cAMP content and smooth-muscle relaxation induced by beta-adrenergic agonists.

All known effects of cAMP in mammalian biological systems are thought to be due to the cAMP activation of cAMP-dependent protein kinase (Krebs and Beavo, 1979). It is, therefore, logical to suppose that the relaxation of smooth muscle by beta-adrenergic-receptor stimulation may be mediated through the cAMP activation of cAMP-dependent protein kinase as well as the phosphorylation of key protein(s) involved in the contractile process. The phosphorylation of purified smooth-muscle MLCK by cAMP-dependent protein kinase causes a change in the enzymatic properties of MLCK with an increase in the Ca^{2+} - calmodulin concentrations required for activity. This basic observation provides strong support for an important role for cAMP in mediating smooth-muscle relaxation (cf. Kamm and Stull, 1985 and see Figure 1).

Given the central role that Ca^{2+} plays in eliciting contractions in smooth muscle, it has been proposed that cAMP formation may lead to a decrease in Ca^{2+} availability to contractile proteins (Kroeger, 1983). A decrease in sarcoplasmic Ca^{2+} concentrations mediated by beta-adrenergic receptors has been demonstrated. The primary mechanisms for this are increased Ca^{2+} sequestration into intracellular storage sites (Casteels and Raeymaekers, 1979; Mueller and Van Breeman, 1979; Itoh et al., 1982), decreased Ca^{2+} influx into smooth-muscle cells (Meisheri and Van Breeman, 1982) and increased

Ca^{2+} efflux from them (Bulbring and den Hertog, 1980; Scheid and Fay, 1984). Furthermore, isoproterenol has been shown to activate Na^+ - K^+ pumping in isolated smooth-muscle cells (increased Ca^{2+} efflux) and cAMP-dependent protein kinase stimulates the Na^+ - K^+-dependent ATPase activity in membrane fragments from these cells (Scheid et al., 1979). These data as well as the recent demonstration of beta-adrenergic stimulation of K^+ influx and Ca^{2+} efflux (Scheid and Fay, 1984) indicate that isoproterenol-induced relaxation may be mediated in part by increasing Na^+ - K^+ pump activity, with a concomitant decrease in contractility via increased Ca^{2+} extrusion by the Na^+ - Ca^{2+} exchange mechanism. The relationship, if any, between cAMP and intracellular Ca^{2+} levels remains unclear.

During the past decade, an association has also been found between smooth-muscle contractility and the metabolism of certain membrane phospholipids such as phosphatidylinositol (PI) (Lapetina et al., 1976; Villalobos-Molina and Garcia-Sainz, 1983; Holzer and Lippe, 1985) and thus interference with PI metabolism and a consequent lowering of cytosolic calcium may account for hormonal relaxation of smooth muscles. Due to the extensive physiological and pharmacological diversity of smooth-muscle cells, it seems likely that the relative importance of these mechanisms may vary from one type of smooth muscle to another.

Recent evidence suggests that agonist-activated PI turnover is initiated by polyphosphoinositides, which are very rapidly hydrolyzed in resonse to agonists (Berridge, 1983; Creba et al., 1983; Michell, 1983). The enzyme catalyzing this hydrolysis is phospholipase-C

resulting in the generation of two second messengers, namely inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Gill, 1985). IP_3 increases cytosolic Ca^{2+} by releasing Ca^{2+} from sarcoplasmic reticulum. DAG can activate the enzyme, protein kinase-C which subsequently phosphorylates certain membrane proteins (Berridge and Irvine, 1984). Similarities in the signal transuction mechanisms of adenylate cyclase and polyphosphoinositides have recently become evident. It has been shown that islet activating protein (IAP, pertussis toxin) suppresses leukotriene B_4-, and the chemotactic peptide, formylmethionylleucyl-phenylalanine-, evoked [^{3}H]inositol-triphosphate accumulation in rabbit neutrophils (Bradford and Rubin, 1983). This suggests that guanine nucleotide binding protein (Ni), which is ADP-ribosylated and inactivated by IAP, may mediate coupling between both an adenylate cyclase-linked receptor and calcium mobilizing receptors that are linked to the metabolism of polyphosphoinositides.

1.3.7.3 Effect of adenosine derivatives on the vasculature

Adenosine has been shown to relax many anatomically different vascular smooth muscles such as canine coronary artery (Schnaar and Sparks, 1972), hog carotid artery (Herlihy et al., 1976), rabbit coronary artery (Gellai et al., 1973), rat aorta (Cohen and Berkowitz, 1974), guinea-pig aorta (Collis and Brown, 1983), bovine coronary artery, rabbit aorta, rabbit carotid artery (Baer and Vriend, 1985; Mustafa and Askar, 1985), rat brain microvessels (Schutz et al., 1982; Huang and Rorstad, 1982) and cat cerebral artery (Edvinsson and Fredholm, 1983). In all those systems where rank order of potency of

adenosine derivatives has been studied, NECA is more potent than R-PIA, and thus relaxation has been tentatively attributed to A_2 receptors (Kusachi et al., 1983). The relaxation of rabbit renal artery seems to be mediated through an A_1-type receptor (R-PIA>NECA) (Baer and Vriend, 1985). Furthermore, adenosine has been reported to contract isolated tail artery of rat and this effect is probably indirectly mediated through the release of serotonin (Brown and Collis, 1981).

1.3.7.4 Effect of adenosine derivatives on non-vascular smooth muscle

A variety of non-vascular smooth muscle systems relax in response to adenosine and its N^6- and 5'-substituted derivatives. On the basis of the agonist rank order potency, the receptors in these tissues have been typified as A_1 or A_2. The various systems studied are rabbit small intestine (A_1; Baer and Vriend, 1985), guinea-pig ileum (A_2; Gustafsson et al., 1985), rabbit taenia coli (A_2; Baer and Vriend, 1985), guinea-pig taenia caeci (A_2; Baer and Muller, 1983; Burnstock et al., 1984) and guinea-pig trachea (A_2; Brown and Collis, 1982). Thus, on the basis of the rank order potency, there seems to be no consistency to the type of adenosine receptor mediating relaxation of various vascular and non-vascular smooth-muscle systems. However, irrespective of the type of receptor involved, the relaxation response is blocked by methylxanthines. In isolated small intestine of rabbit N^6-derivatives of adenosine such as PIA and HPIA are the most potent relaxants (ED_{50}=37 and 64 nM, respectively) and R-PIA is about 27-fold more potent than S-PIA. 2-Chloroadenosine and NECA have

lower potencies; ED_{50}=510 and 110 nM, respectively (Baer and Vriend, 1985). This rank order of potency is typical of that seen at A_1 (adenylate cyclase-inhibitory) receptors in systems such as brain. However, an effect of these compounds on adenylate cyclase activity in the particulate fraction from these tissues is not demonstrable (Muller, 1985). These relaxations are, however, sensitive to blockade by theophylline, 8-SPT and 8-phenyltheophylline in that order of increasing potency (Baer, unpublished data).

1.3.7.5 Mechanism of adenosine-induced smooth muscle relaxation

1.3.7.5.1 Involvement of cAMP

The cAMP hypothesis described in section 1.3.7.2 is based on the observation that adenosine-induced relaxation parallels cAMP accumulation in bovine coronary artery (Kukovetz, 1978; Muller, 1985). Furthermore, Jonzon et al. (1985) have reported adenosine-mediated increases in cAMP levels in cultured arterial smooth-muscle cells and the observed rank order potency of agonists (NECA>adenosine>R-PIA) is consonant with an A_2 receptor-mediated effect. On the other hand, dissociation between adenosine-mediated relaxation and cAMP content in coronary arteries (Herlihy et al. 1976; Verhaeghe, 1977) and in rabbit intestine (McKenzie et al., 1977a,b) has also been reported. However, since then several reports of adenosine-sensitive adenylate cyclase activity have appeared. Adenosine-stimulated adenylate cyclase activity has been demonstrated in membranes from primary myocyte cultures of rat aorta (Anand-Srivastava et al., 1983), cultured

smooth-muscle cells from mesenteric artery (Anand-Srivastava and Franks, 1985), and rat cerebral-cortical microvessels (Schutz et al., 1982; Huang and Rorstad, 1983). In all these systems, the rank order potency for stimulation of the adenylate cyclase conforms to that seen at an A_2 receptor.

In this context, it is of interest that forskolin acts as an effective relaxant in a number of smooth muscle preparations, e.g., beef coronary artery and isolated small intestine from rabbit (Muller and Baer, 1983a,b). Potentiation of the vasoactivity of adenosine by forskolin has also been demonstrated in open-chest dogs (Kusachi et al., 1984). If forskolin does not affect membrane potential and Ca^{2+} fluxes, this observation would support the hypothesis that cAMP is a relaxant stimulus, at least in vascular smooth muscle. Furthermore, adenosine-induced stimulation of cAMP-dependent protein kinase activity has now been measured in coronary arterial smooth muscle (Silver et al., 1984) and this lends further support to the involvement of cAMP in adenosine-induced relaxation of vascular smooth muscle.

Although adenosine-induced smooth-muscle relaxation may appear to be mediated through A_2 receptors (cyclase stimulating), the rank order potency of adenosine analogs in all isolated smooth-muscle preparations does not conform to that for A_2 receptors. Besides A_2 receptors and P-site, smooth-muscle cells may possess A_1 receptors (cyclase inhibiting). In this respect, it is interesting that R-PIA inhibits forskolin-stimulated cAMP accumulation in cultured arterial smooth muscle cells (Jonzon et al., 1985). In the same system, NECA

failed to decrease forskolin-stimulated cAMP levels although both NECA and R-PIA stimulated the accumulation of cAMP compared with basal levels (in the absence of forskolin). This suggests the presence of both A_1 and A_2 receptors on smooth muscle cells and also an interesting possibility that the A_1 receptor in smooth-muscle cells may be different from that present in brain and fat cells.

Collis and Brown (1983) have considered the involvement of the P site in adenosine-mediated relaxation of guinea-pig aorta. This is further supported by observations that 6-hydroxylamino-purine riboside, an effective relaxant of smooth muscle, also causes inhibition of adenylate cyclase activity in smooth muscle (McKenzie et al., 1977b) and cardiac membranes (Baer and McKenzie, 1973), presumably via the P site. However, implications of the P site in relaxation, would contradict the basic hypothesis of the involvement of cAMP in relaxation.

Although increases in cAMP levels mediated by adenosine agonists and in adenylate cyclase activity have been reported in various vascular smooth-muscle systems, there is little evidence for these in nonvascular smooth muscle such as intestinal muscle. The adenosine receptor in rabbit intestinal smooth muscle appears to be different from an A_2 receptor. There are five reasons for this conclusion. 1) The rank order of relaxant potency of various adenosine derivatives conforms to that at an A_1 receptor (Baer and Vriend, 1985), rather than at A_2. However, inhibition of adenylate cyclase would contradict the basic hypothesis of cAMP involvement in relaxation (section 1.3.9.2). 2) There is lack of correlation between cAMP concentrations

and intestinal smooth-muscle relaxation (McKenzie et al., 1977b; Baer and Paton, 1978). 3) The phosphodiesterase inhibitor, RO-20-1724, does not potentiate 2-chloroadenosine-induced relaxation, although isoproterenol responses are potentiated (Muller and Baer, 1983b). 4) Adenylate cyclase activity is not stimulated by adenosine derivatives (Muller, 1985). 5) Differential effects of certain adenosine derivatives on smooth muscle and on the classical adenylate cyclase-coupled A_2 receptor system present in murine neuroblastoma cell membranes have been observed. For example, N^6-gamma-trimethylammonio-propyladenosine stimulates neuroblastoma adenylate cyclase activity but it does not cause smooth muscle relaxation (Baer et al., 1983).

In guinea-pig ileum, adenosine derivatives inhibit the contractions induced by direct (electrical) muscle stimulation with an order of potency NECA>R-PIA>S-PIA (Gustafsson et al., 1985). This rank order is typical of that seen at an A_2 receptor. However, only NECA-induced inhibition of contractions is enhanced by the phosphodiesterase inhibitor K62.711. Thus, there is no direct evidence to implicate cAMP in adenosine receptor-mediated relaxation of the intestinal smooth muscle, although it has been proposed that catecholamine-mediated relaxation of rabbit intestine is mediated by cAMP (Anderson, 1972). On the other hand, forskolin which also relaxes intestinal tissue stimulates adenylate cyclase activity in the particulate fraction (Muller and Baer, 1983b). The question of the involvement of adenylate cyclase in adenosine-mediated intestinal smooth muscle relaxation therefore remains controversial.

1.3.7.5.2 Involvement of Calcium

Like beta-adrenergic agonists, adenosine has been shown to modulate Ca^{2+} fluxes in smooth muscles. From experiments with high K^+ solutions and various Ca^{2+} concentrations, Schnar and Sparks (1972) concluded that relaxation of coronary arteries by adenosine occurred without changes in the electrical state of the membrane and that the nucleoside acted by altering Ca^{2+} fluxes. Both adenosine and verapamil have been shown to inhibit the slow inward Ca^{2+} current in coronary arteries (Harder et al., 1979). Furthermore, adenosine inhibits K^+ depolarization-induced $^{45}Ca^{2+}$ uptake by cultured smooth-muscle cells from rat aorta and porcine carotid artery (Fenton et al., 1982).

In guinea-pig taenia coli, adenosine hyperpolarizes the smooth-muscle membranes (Axelsson and Holmberg, 1969; Ferrero and Frischknecht, 1983), which inactivates the spike mechanism and consequently produces relaxation. Recently, adenosine has been shown to increase the fractional rate of $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-labelled guinea-pig taenia coli strips (Frischknecht and Ferrero, 1984).

Thus, stimulation of Ca^{2+} extrusion and/or inhibition of Ca^{2+} influx may take part in the mechanism of smooth-muscle relaxation by adenosine. Furthermore, an increase in an internal store of Ca^{2+} may contribute to relaxation of the taenia (Frischknecht and Ferrero; 1985), as has been shown with beta-adrenergic agonists (Casteels and Raeymaekers, 1979).

Changes in Ca^{2+} fluxes in response to adenosine have also been reported in systems other than smooth muscle. Adenosine has been shown

to depress Ca^{2+}-dependent potentials in rat hippocampal pyramidal cells (Proctor and Dunwiddie, 1983) and reduce the influx of $^{45}Ca^{2+}$ into K^+-depolarized brain synaptosomes (Ribeiro et al., 1979). In view of the fact that the entry of Ca^{2+} into the axon terminal is an essential step for release of the neurotransmitter (Katz and Miledi, 1968), it is possible that adenosine may inhibit transmitter release by decreasing the availability of Ca^{2+}. Furthermore, adenosine receptor agonists exhibited different abilities to inhibit K^+-evoked Ca^+ uptake in rat brain cortical synaptosomes, which were correlated with their affinities for adenosine receptors in brain, and this effect is sensitive to blockade by theophylline (Wu et al., 1982).

That adenosine may act by modulating Ca^{2+} fluxes is further supported by observations from peripheral nerve terminals. Besides its effect on the release of neurotransmitters in brain, adenosine also depresses peripheral neurotransmission from autonomic and motor nerve terminals. Adenosine depresses presynaptically the release of norepinephrine from rabbit, guinea-pig and rat heart (Wakade and Wakade, 1978; Hedqvist and Fredholm, 1979; Khan and Malik, 1980), dog basilar artery (Muramatsu et al., 1981), rabbit pulmonary artery (Husted and Nedergaard, 1981), dog saphenous vein (Verhaege et al., 1977), rat portal vein (Wakade and Wakade, 1978), rat and guinea-pig vas deferens (Clanachan et al., 1977; Paton et al., 1978; Hedqvist and Fredholm, 1976) and rat anococcygeus muscle (Stone, 1983). Adenosine also depresses the release of acetylcholine from parasympathetic nerve terminals of guinea-pig ileum (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976; Paton, 1981), and from motor nerve terminals such as rat

diaphragm phrenic nerve (Ginsborg and Hirst, 1972) and frog pectoris muscle nerve (Silinsky, 1984). The presynaptic effect of adenosine is dependent on the level of extracellular Ca^{2+} (Ginsborg and Hirst, 1972; Dowdle and Maske, 1980; Hedqvist and Fredholm, 1979; Vizi and Knoll, 1976) which is taken as evidence for a Ca^{2+}-dependent mechanism (Westfall, 1977).

In summary, alteration of Ca^{2+} fluxes by adenosine in various systems has been reported. Whether these effects are direct or secondary to an increase in the cAMP levels is not yet clear. It is possible that there are differences between adenosine receptors in various systems from different species and these receptors may be linked to different effector systems.

1.4 Radioligand binding to adenosine receptors

1.4.1 General

The concept of the existence of adenosine receptors has been developed from several independent lines of research. The initial study of the molecular mechanisms of adenosine action in relation to cAMP generating systems has been followed by the use, during the last decade, of radioligand binding techniques to further characterize the receptors. In most studies performed using membrane preparations containing adenosine-sensitive adenylate cyclase, A_1 receptors have been labelled. The earliest attempts to identify adenosine receptors by radioligand binding studies were performed with tritium labelled adenosine using fat-cell membranes (Malbon et al., 1978), rat brain

membranes (Schwabe et al., 1979), and ^{14}C-labelled adenosine in dog coronary and carotid arteries (Dutta and Mustafa, 1980). Because of the extensive metabolism of adenosine (under the conditions of receptor binding assays) and its affinity for the intracellular P site and the nucleoside transporter, several drawbacks of labelled-adenosine binding became apparent (see review by Schwabe, 1983). The advent of metabolically stable derivatives of adenosine that also have much lower affinity at the P site and nucleoside transporter site, allowed the direct study of physiologically important adenosine receptors. Agonist radioligands such as [^{3}H]-2-chloroadenosine (Williams and Risley, 1980; Wu and Phillis, 1982), [^{3}H]-N^6-R-(2-phenyl)isopropyladenosine ([^{3}H]R-PIA; Schwabe & Frost, 1980), [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]CHA; Burns et al., 1980) and [^{3}H]-5'-N-ethylcarboxamidoadenosine ([^{3}H]NECA) (Yeung and Green, 1983) were introduced and used successfully in the identification of adenosine receptors.

The binding of these radioligands in the presence of adenosine deaminase appears to satisfy all the criteria for ligand binding to adenosine receptors. The specific binding of all new radioligands in membranes treated with adenosine deaminase was two- to four-fold higher than in untreated membranes, whereas nonspecific binding was not changed (Daly, 1983). Furthermore, it is clear that treatment with adenosine deaminase increases binding specificity. This is supported by the observation that in untreated rat brain membranes, [^{3}H]-chloroadenosine binding was displaced by inosine, hypoxanthine and adenine (Wu et al. ,1980), whereas these compounds were not active

at [^{3}H]-chloroadenosine binding sites in the treated membranes (c.f. Daly, 1980). The binding of these radoligands is sensitive to alkylxanthines like theophylline, caffeine and IBMX. In addition, the P site agonist 2',5'-dideoxyadenosine does not substantially inhibit the binding of the radioligands (see reviews by Schwabe, 1981 and Daly, 1985).

1.4.2 Subtype specificity

Londos et al. (1980) classified R-PIA as subtype selective for A_1 receptors and NECA as subtype selective for A_2 receptors. However, Yeung and Green (1984) have recently shown that [^{3}H]NECA binds to A_1 receptors with high affinity in rat hippocampus membranes. Using NEM-pretreated striatal membranes, these authors also demonstrated that [^{3}H]NECA bound to two different (high and low affinity) sites in untreated membranes (shallow displacement curves for CHA, R-PIA and 2-chloroadenosine), whereas the binding using NEM-pretreated membranes revealed a single population of sites with the characteristics of an A_2 receptor. These authors have also reported both inhibition and stimulation of adenylate cyclase activity by CHA and NECA in striatal membranes, under different assay conditions. Thus, NECA, PIA and CHA appear to be only relatively selective at the two types of adenosine receptors.

1.4.3 A_1-Receptor binding

First reports of successful A_1 receptor binding came from studies utilizing labelled purine-modified compounds such as [^{3}H]R-PIA,

[^{3}H]CHA, and [^{3}H]2-chloroadenosine as radioligands in brain from various species (see reviews by Daly, 1982, 1983, and Schwabe, 1981, 1983; Williams, 1983b). Binding affinity was high (K_D=0.3 - 6 nM). The order of potency of agonists in displacement studies was R-PIA> NECA>2-chloroadenosine>S-PIA and, in general, at least a 10-fold difference in the affinity of the enantiomers of PIA was observed. This order of potency is similar to that seen in A_1 receptor-mediated inhibition of adenylate cyclase. The B_{max} value ranged between 200 - 800 fmols/mg protein (Table 1). The B_{max} values in rat brain membranes were high using [^{3}H]R-PIA probably because binding was performed at 37°C compared with [^{3}H]CHA binding in other species at 20 - 25°C. The difference in B_{max} values may therefore be due to the temperature dependence of [^{3}H]R-PIA binding and not to labelling of a different population of adenosine receptors (Murphy [illegible]nyder, 1982). The order of potency of antagonists was 8-phenyltheophylline> IBMX>SPT>theophylline>caffeine>theobromine (Bruns et al., 1980). The binding of these radioligands to brain A_1 receptors, thus, meets the requirement for the specificty of binding to adenosine receptors.

Other radiolabelled ligands used in A_1 receptor studies are [^{3}H]NECA and the antagonist radioligand [^{3}H]-1,3-diethyl-8-phenylxanthine ([^{3}H]DPX). [^{3}H]NECA binds to A_1 receptors in rat hippocampus with lower affinity than other radioligands such as [^{3}H]R-PIA and [^{3}H]CHA, which is consistent with the lower potency of NECA than PIA or CHA at A_1 receptors mediating inhibition of adenylate cyclase (Londos et al., 1980).

Table 1. Binding of radioligands to adenosine receptors in brain

Radioligand	Membrane Preparation	K_D (nM)	B_{max} (fmols/mg)	Reference
[^{3}H]2-chloroadenosine	Rat brain	2.3	476	Wu et al., 1980
		1.3, 16	207, 380	Williams and Risley, 1980
	Bovine atria	1.3, 176	75, 3700	Michaelis et al., 1985
[^{3}H]CHA	Rat brain	0.7, 2.4	230, 120	Patel et al., 1982
		2.3	301	Geiger et al., 1984
	Rat cortex	0.4, 4.2	159, 329	Marangos et al., 1983
	Rat hippocampus	1.8	547	Yeung and Green, 1983
	Calf cortex	0.29	310	Murphy and Snyder, 1982
	Human cortex	5.2	92	Murphy and Snyder, 1982
	Bovine cortex	0.3, 1.8	340, 200*	Bruns et al., 1980
		0.4	340	Gavish et al., 1982
		0.5	410	Goodman et al., 1982
	Guinea-pig brain	5	206	Goodman et al., 1982
		6	370*	Bruns et al., 1980

. . . Cont'd

Radioligand	Membrane Preparation	K_D (nM)	B_{max} (fmols/mg)	Reference
[^{3}H]R-PIA	Rat brain	5	810	Schwabe and Trost, 1980
	Rat cortex	1.4	740	Lohse et al., 1984
	Rat hippocampus	4.2	783	Green, 1984
	Guinea-pig cortex	1.8	169*	Muprhy and Snyder, 1982
	Rabbit cortex	0.9	230*	Muprphy and Snyder, 1982
[^{3}H]NECA	Rat hippocampus	12.3	510	Yueng and Green, 1984
[^{3}H]DPX	Rat hippocampus	196	923	Green, 1984
	Bovine brain	5	1000*	Bruns et al., 1980
	Calf cortex**	0.65 (Ki)		Murphy and Snyder, 1982
	Rabbit cortex**	5.1 (Ki)		Murphy and Snyder, 1982
	Guinea-pig cortex**	-		Murphy and Snyder, 1982
	Human cortex**	-		Murphy and Snyder, 1982

*B_{max} recalculated on the basis 1 g brain tissue equivalent to 100 mg protein

**binding at 0°C (rest of the binding at 23-37°C)

[^{3}H]DPX binds to brain adenosine receptors with still lower affinity and exhibits considerable species difference (Table 1).

Although all N^6-substituted adenosine derivatives exhibit similar affinity in brain from various species such as rat, rabbit, guinea pig, calf and human, the antagonist DPX manifests considerable difference in affinity (Ki) for [^{3}H]CHA-labelled A_1 receptors in these species. DPX has decreasing affinity for A_1 receptors in this order in brain cortex of the following species: calf, rat, rabbit, human and guinea pig, with affinity in the last >300-fold less than in calf cortex. Furthermore, very little specific [^{3}H]DPX binding is detected in human and guinea-pig brain and this limited amount is inhibited poorly by R-PIA and xanthines. Adenosine-insensitive binding sites for [^{3}H]DPX have also been reported in membranes from cultured glia and neurons of chick embryo brain (Barnes and Thanpy, 1982). As the affinity of adenosine antagonists decreases at higher temperatures, variation in temperature may explain the lower affinities of DPX compared with the agonists at A_1 receptors. However, it does not account for the failure of [^{3}H]DPX to label A_1 receptors in human and guinea-pig brain, even at 0°C (Murphy and Snyder, 1982; Marangos et al., 1983). It thus appears that, at least for DPX, A_1 receptors in the brain of various species may be heterogeneous.

[^{3}H]R-PIA and [^{3}H]CHA have since proven extremely useful in binding studies with adenosine receptors and these radioligands have also been used to characterize A_1 receptors in systems other than brain. Their affinity in some peripheral systems such as heart and testis is similar to that in brain (Table 2). However, in certain

Table . . Binding of radioligands to adenosine receptors in peripheral systems

Radioligand	Membrane Preparation	K_D (nM)	B_{max} (fmols/mg)	Reference
[^{3}H]CHA	Guinea-pig myenteric plexus	1.8	139	Williams and Valentine, 1985
	Rat testis	2	*200	Murphy and Snyder, 1982
	Rat spinal cord	2.3 - 2.6	123 - 170	Geiger et al., 1984
[^{3}H]R-PIA	Rat fat cells	6	1900	Trost and Schwabe, 1982
	Chick ventricular myocardium	3 - 5	164	Hosey et al., 1984

*B_{max} recalculated on the basis of 1 g brain tissue equivalent to 100 mg protein

Binding measured at 20 - 37°C

other peripheral systems such as rat liver, kidney, pancreas, and stomach, no specific binding was detectable with [^{3}H]CHA (Murphy and Snyder, 1981). This could reflect either an absence of high-affinity receptors in these systems or a low density of receptors that would not be measurable with the relatively low specific radioactivity of a tritium-labelled radioligand. In these systems, therefore, development of radioligands with high specific activity such as [^{125}I]-labelled compounds could prove useful for the measurement of adenosine receptors (Baer and Paton, 1978).

1.4.4 A_2 Receptor binding

Most A_2 receptor binding has been performed utilizing [^{3}H]NECA. A_2 receptors have been satisfactorily characterized with [^{3}H]NECA in NEM-pretreated membranes from rat striatum (Yeung and Green, 1983, 1984). Various agonists displace [^{3}H]NECA binding with the rank order potency, NECA>R-PIA>CHA>S-PIA, as would be expected for binding to an A_2 receptor. However, [^{3}H]NECA binding in other systems such as rat liver membranes (Schutz et al., 1982), calf thymocytes (Ukena et al., 1982) and human platelets (Huttermann et al., 1984), reveals characteristics not consonant with binding to A_2 receptors (Table 10). In all of these systems, including rat striatum, adenosine derivatives stimulate the activity of adenylate cyclase in the particulate fractions and NECA is more potent in this respect than PIA or CHA. Thus, [^{3}H]NECA may not be a suitable ligand for binding studies in some peripheral systems that possess an A_2-receptor.

Other radioligands such as [^{3}H]R-PIA used in human platelet

membranes (Huttermann et al., 1984), and [^{3}H]CHA in rat liver membranes (Schutz et al., 1982) also failed to bind A_2 receptors with the required specificity. Thus, characterization of A_2 receptors in some peripheral systms by binding studies must await the development of new radioligands.

1.4.5 Modulation of radioligand binding to adenosine receptors

Guanine nucleotides decrease the affinity of agonist binding to both A_1 and A_2 receptors (Limbird, 1981; Cooper, 1982), while not affecting antagonist binding to them (Goodman et. al., 1982). This effect resembles the actions of numerous neurotransmitter and hormone receptors that are associated with adenylate cyclase activity such as alpha$_2$-adrenergic (U'Prichard and Snyder, 1978), D_2-dopamine (Creese et al., 1979), serotonin (Peroutka et al., 1979), H_1-histamine (Chang and Snyder, 1980), and opiate receptors (Childers and Snyder, 1980). As well, physiological concentrations of sodium decrease the affinity of agonists without affecting antagonist affinity (Goodman et al.,1982) just as at histamine H_1 (Chang and Snyder, 1980), opiate (Pert and Snyder, 1974) and alpha$_2$-adrenergic (Greenberg et al., 1978) receptors. The effect of Na^+ is seen only at A_1 and not A_2 adenosine receptors (Limbird, 1981; Cooper, 1982) and its physiological relevance is not clear. By contrast, divalent cations such as Mn^{2+}, Mg^{2+}, and Ca^{2+} (at endogenous concentrations in brain) increase the affinity and B_{max} of agonist binding at A_1 receptors (Goodman et al., 1982), without affecting antagonist binding and this effect is seen with other receptor systems too such as opiate (Pasternak et al.,

1975), alpha$_2$-adrenergic (U'Prichard and Snyder, 1978) and D_2-dopamine (Usdin et al., 1980).

On the basis of the differential influence of guanine nucleotides on agonist and antagonist binding, Lohse et al. (1984) have proposed that the A_1 receptor in rat brain membranes exists in two different affinity states that are distinguishable by agonists, but not by antagonists. The relative proportions of receptor in the two affinity states also depends upon temperature; at lower temperatures fewer receptors are in the high affinity state. Further differences between the two states are revealed by thermodynamic analysis of the binding of agonists to them. Thus, agonist binding to the high affinity state is associated with an increase in enthalpy and is entropy-driven (i.e. affinity is higher at higher temperatures) whereas agonist binding to the low affinity state, like the antagonist binding is largely enthalpy driven with an additional entropy component that also characterizes the binding of antagonists (Lohse et al., 1984; Murphy and Snyder, 1982). Similar observations have been made with opiate receptors (Simantov et al., 1977).

1.4.6 Radioligand binding to smooth muscle adenosine receptors

The initial studies of radioligand binding to smooth-muscle adenosine receptors utilized labelled adenosine and were mostly performed in the presence of an inhibitor of adenosine deaminase (Table 3).

[^{3}H]NECA and [^{3}H]2-chloroadenosine have now been used to characterize the receptors from the smooth muscle of rat brain

Table 3. Binding of radioligands to smooth-muscle adenosine receptors

System	Radioligand	K_D (μM)	B_{max} (pmol/mg)	Rank Order of Potency	References
Dog carotid artery	[^{14}C]AR	1.34	140	AR>2-ClAR>>ATP	Dutta & Mustafa (1980)
Dog coronary artery	[^{14}C]AR	0.83	-	AR>>ATP	Dutta & Mustafa (1980)
Hog carotid artery*	[^{3}H]AR	0.23 4.30	1.7 21.6	ATP>AR>2-ClAR> NECA>>DDAR>>R-PIA[a]	Schutz & Brugger (1982)
Bovine coronary artery*	[^{3}H]AR	0.17 2.60	1.2 6.6	ATP>>2-ClAR>>R-PIA> Theophylline	Ollinger & Kukovetz (1983)
Rat brain microvessels	[^{3}H]NECA	0.019 2.50	0.084 5.20	NECA>>DDAR>S-PIA> 2-ClAR>AR>R-PIA	Schutz et al. (1982)
Human placenta	[^{3}H]2-ClAR	0.056	1.10	2-ClAR>>NECA>> S-PIA>R-PIA=CHA	Fox & Kurpis (1983)
Mouse cerebral vessels	[^{3}H]2-ClAR	0.033	0.283	-	Beck et al. (1984)

*in the presence of EHNA/dcF

microvessels and human placenta, respectively (Table 3). However, once again the specificity of the binding as measured by rank order of potency of various agonists does not conform to what is observed at classical A_1 or A_2 receptors, such as those in brain. The most striking feature of nonspecificity is the effective displacement of [^{3}H]NECA binding by 2',5'-dideoxyadenosine. Furthermore, N^6-derivatives of adenosine such as R-PIA and CHA are about 1000-fold less potent than 2-chloroadenosine at [^{3}H]2-chloroadenosine binding sites. Autoradiographic localization of [^{3}H]NECA binding sites in guinea-pig small intestine reveal specific sites on both longitudinal and circular muscle (Buckley and Burnstock, 1983). However, 8-phenyl-theophylline does not displace [^{3}H]NECA binding. In systems such as hog carotid artery and bovine coronary artery, 2-chloroadenosine, R-PIA, and theophylline were weaker competitors of binding than adenosine, which disagrees with the pharmacological findings (Ollinger and Kukovetz, 1983; Schutz and Brugger, 1982). No detailed studies of specificity were performed in dog carotid and coronary arteries (Dutta and Mustafa, 1980).

In summary, the known radioligands for adenosine receptors such as [^{3}H]AR, [^{3}H]R-PIA, [^{3}H]CHA, [^{3}H]2-chloroadenosine, and [^{3}H]NECA have proven not useful in characterization of A_2 receptors in some systems such as various vascular smooth muscles, platelets, liver, and thymocytes, although in most of these systems adenylate cyclase activity that can be stimulated by adenosine has been demonstrated with the characteristics of an A_2 receptor. However, binding studies utilizing these radioligands have not been reported in certain other

peripheral systems such as small intestinal muscle where the receptor may not be of the typical A_2 type (i.e., where adenylate cyclase stimulation was not demonstrable). Such studies constitute the major part of the work presented in this thesis.

1.5 Research Objective

As stated earlier, adenosine derivatives are potent relaxants of isolated small intestine from rabbit (Baer and Vriend, 1985). Smooth-muscle relaxation is thought to be mediated through increases in intracellular cyclic AMP levels and there is good evidence for this from studies of beta-adrenergic agonists (Kamm and Stull, 1985). Since adenosine derivatives stimulate adenylate cyclase activity through A_2 receptors in various systems such as striatum, platelets, and liver, and in cerebral smooth muscle, it was suspected that smooth-muscle relaxation in response to these compounds might involve A_2 receptors. However, adenosine-stimulated adenylate cyclase activity in the smooth-muscle membranes of rabbit small intestine is not demonstrable (Muller, 1985). This suggests that the adenosine receptor in intestinal-smooth muscle may be different from the well characterized adenylate cyclase-coupled A_2 receptor.

As we could draw no definite conclusions from the structure-activity data and adenylate cyclase assays about the type of adenosine receptor in the gut muscle, we decided to try another possible route of enquiry: that of direct measurement by a radioligand binding reaction. Furthermore, guanine nucleotides are known to modulate the binding of agonist ligands to their receptors (section 1.3.5). If

guanine nucleotides did affect the binding of these radioligands to the receptors, it would provide indirect evidence that adenosine receptors in smooth muscle are linked to the adenylate cyclase system.

Two radioligands that are relatively specific for A_1 and A_2 receptors, ([^{3}H]R-PIA) and [^{3}H]NECA), respectively, were employed as probes. A new radioligand with an [^{125}I] label, [^{125}I]N^6-R-(3-iodo,4-hydroxyphenyl)isopropyladenosine ([^{125}I]R-IHPIA), was developed and then used to investigate the receptors in rabbit gut after initial characterization of this radioligand in rat brain membranes. In addition, two relatively little studied 5'-deoxy derivatives of adenosine, 5-deoxy-5'-chloroadenosine (CDA) and 5'-deoxy-5'-methylthioadenosine (MTA), were employed to investigate the receptors in rabbit gut.

2. METHODS AND MATERIALS

2. METHODS AND MATERIALS

2.1 Chemical Synthesis

2.1.1 Synthesis of R-HPIA and S-HPIA

Isomers of p-hydroxyamphetamine (11 mg of the (+) form and 7 mg of the (-) form, free bases, Appendix II) were each reacted with 40 mg 6-chloropurine riboside in 0.2 ml anhydrous dimethylsulfoxide at 70°C for about 4 h. The solvent was removed overnight under vacuum (oil pump) and the residue dissolved in 1 ml of ethanol with heating. After cooling, excess 6-chloropurine riboside crystallized and was removed by filtration. The filtrate was subjected to thin-layer chromatography (TLC) on silica gel (10 x 20 cm, 0.2 mm thickness) using ethylacetate: acetone (4:1 v/v) as the solvent. Separated bands representing the respective HPIA isomers were located under UV light and each was scraped off the plate and eluted with methanol. After removal of methanol on a rotary evaporator, the residue was dissolved in ethanol and stored at 4°C. The UV spectrum and chromatographic behaviour of each enantiomers were identical to those of an authentic mixture of HPIA enantiomers. The yields were calculated on the basis of UV absorption at 270 nm (ε_{270}=16800 M^{-1} cm^{-1}) to be 8.6 mg (30%) S-HPIA and 4.6 mg (24%) R-HPIA. The ethanol solution of each isomer was then evaporated and the residue dissolved in dimethyl sulfoxide to obtain stock concentrations of 50 mM which were stored at 4°C.

2.1.2 Synthesis and Purification of IHPIA

HPIA (40 mg, 0.1 mmol) was dissolved in 1 ml dimethylformamide and diluted with 2 ml of 0.5 M potassium phosphate (pH 7.5) and 5 ml H_2O.NaI (28 mg, 0.1 mmol) in 0.5 ml H_2O was added followed by dropwise addition of 15 mg (0.1 mmol) chloramine-T in 0.5 ml H_2O over a 1 - 2 min period. The solution was concentrated on a rotary evaporator at 30°C, frozen, and freeze-dried overnight.

The sirupy residue was dissolved in methanol and separated on a 2 mm layer of silica gel PF-254 (20 x 40 cm, E. Merck AG). The first development was carried out with chloroform, a second with chloroform/methanol (8:1), with increasing R_f values separating HPIA from IHPIA and the diiodo derivative. Elution of the respective UV-active bands yielded 10 mg (19%) IHPIA and 5 mg (8%) of the presumed diiodo derivative after evaporation. Mass spectrum calculated for $C_{19}H_{22}N_5O_5I$ 527.0666 found 527.0663. The presumed diiodo derivative did not yield a mass spectrum and appeared to be unstable, showing brown discoloration within days.

R-IHPIA was synthesized in the same manner, but using only 10 mg R-HPIA and the reaction mixture scaled down to one quarter. After TLC, the separated band containing IHPIA was scraped off the plate and eluted with methanol. The concentration of the methanol solution obtained (500 µM) was calculated on the basis of UV absorption at 271 nm (ε_{270}=20600 M^{-1} cm^{-1}). The yield, however, was very low.

2.1.3 Radio-iodination of HPIA

HPIA was radio-iodinated by the procedure of Hunter and Greenwood (1962) as follows: 5 µl of 10 mM HPIA (50 nmoles) in methanol was evaporated under vacuum in a glass tube and 40 µl of 0.3 M potassium phosphate (pH 7.5) added to dissolve the residue. One millicurie of carrier-free $Na^{125}I$ (0.5 nmoles; 1 Ci=37 GBq) was added followed by 5 µl of freshly prepared chloramine-T (0.17 mg/ml in H_2O). After 1.5 min the reaction was stopped by the addition of 500 µl of sodium metabisulphite (1 mg/ml in 1 M acetic acid). The mixture was extracted with 2 ml and then 1 ml of ethylacetate, and the combined extracts were reduced to about 0.1 ml under vacuum and stored at -20°C.

2.1.3.1 Identification of the product of radio-iodination

Aliquots of the extracts were chromatographed on (1), a 2 mm layer of silica gel PF-254 and developed with methylene chloride and methanol (9:1) and (2), polyethylenimine-impregnated cellulose thin layers (PEI, Macherey-Nagel Company) and developed with 0.25 M LiCl for a length of 10 cm. IHPIA, I_2HPIA, and HPIA were used as markers. The plates were subsequently autoradiographed (Fig. 2).

2.1.3.2 Purification and storage of [^{125}I]-labelled IHPIA

About 100 µl of the ethylacetate extract was applied as a thin streak on a PEI plate (about 4" long), allowed to dry and developed with 0.25 M LiCl. After autoradiography, the radioactive band corresponding to the monoiodoproduct was spotted and cut into small parts and the radioactivity eluted with 95% ethanol (2+1+1 ml). The

4 ml extract was concentrated to 0.5 ml, stored at -20°C and used for about 10 days when solutions were evaporated and again subjected to purification by PEI TLC.

2.2 Adenylate cyclase activity in rat cerebellar membranes

2.2.1 Preparation of a 15,000-xg membrane pellet

Sprague-Dawley rats of both sexes weighing 180-220 g were decapitated and the brains removed. After the cerebellum had been dissected out, the membranes were prepared as described by Schwabe and Trost (1980). The cerebelli were homogenized in 8 volumes of a buffer containing 0.25 M sucrose, 50 mM Tris HCl (pH 7.4) and 1 mM $MgCl_2$, using a Potter Elvehjem homogenizer with teflon pestle. The pestle was electrically driven using a Cafrano stirrer at a setting of 175 and 12 strokes were applied. The homogenate was centrifuged at 1,500 xg for 20 min (2°C). The pellet (P_1) was discarded and the supernatant recentrifuged at 15,000 xg for 15 min (2°C). The pellet (P_2) thus obtained was washed twice using the same buffer but without sucrose and $MgCl_2$. The final washed pellet was redispersed in the sucrose- and $MgCl_2$-free buffer to a concentration of 0.7 - 1.0 mg protein/ml, and the solution divided into aliquots and stored frozen under liquid nitrogen. Protein was determined by the method of Lowry et al. (1951).

2.2.2 Assay of inhibitory adenylate cyclase activity

Adenylate cyclase activity was measured according to the method

of Baer (1975), with certain modifications. α-^{32}P-deoxyATP was used as substrate and PEI TLC to separate the formed radioactive deoxycyclic AMP. The reaction mixture contained 0.1 mM α-^{32}P-deoxyATP, 50 mM Tris HCL (pH 7.4), 2 mM $MgCl_2$, 1 mM deoxycyclic AMP, 10 mM creatine phosphate, 72 U/ml creatine phosphokinase, 10 µM GTP, 5 U/ml adenosine deaminase, 0.4% bovine serum albumin and 7 µg of the membrane protein which had been preincubated with adenosine deaminase (5 U/ml) at 30°C for 10 min before its immediate use in the assay. Incubation was stopped, after 20 min, by adding 10 µl of a solution containing 30 mM each of deoxycyclic AMP, deoxyATP, deoxyAMP and EDTA. About 5 µl of each incubation mixture was applied to PEI plates and then developed with 0.25 M LiCl. After chromatographic separation by PEI TLC, the deoxycyclic AMP and deoxy-ATP/AMP spots were visualized under UV, cut and transferred into vials containing 10 ml of Toluene Scintillant (0.4 g PPO and 4 g POPOP per litre of Toluene), and counted in a Beckman 330 Beta counter. The ratio of counts per minute in the two spots (calculated by computer) provided a measure of the percentage conversion of ATP to cyclic AMP.

2.3 [^{125}I]R-IHPIA binding assays with rat brain membranes

2.3.1 Preparation of a 15,000 xg membrane pellet

Sprague-Dawley rats of both sexes weighing between 180 - 220 g were decapitated and the brains were removed. Membranes were prepared as described (section 2.2.1) for rat cerebellum. The final washed pellet was redispersed in the sucrose-free buffer (containing 1

mM $MgCl_2$) to a concentration of 4 - 6 mg protein/ml, and the solution divided into aliquots and stored frozen under liquid nitrogen. Membranes were used for binding assays within ten days; longer storage tended to reduce their binding capacity. Protein was determined by the method of Lowry et al. (1951).

2.3.2 Standard assay protocol

The membrane protein (100 - 240 µg/tube) was incubated with radioligand and other drugs as indicated, in a final volume of 0.5 ml, containing 50 mM Tris-HCl (pH 7.4) and 1 mM $MgCl_2$. The membranes, which had been preincubated with adenosine deaminase (0.4 U for every 2.5 mg/ml membrane suspension) at 30°C for 10 min just before use, were added last to initiate binding reactions. These were performed at 30°C for 2 h and terminated when 450 µl of the incubations were removed and filtered through wetted, precooled glass fibre filters (Whatman GF/B, 25 mm) followed by two rapid washes each with 5 ml of ice-cold buffer. The filters were counted in a Beckman 8000 gamma-counter which had been pre-calibrated with an ^{129}I probe to yield 73% efficiency. The portion of the binding displaced by 10 µM R-PIA is defined as the specific binding.

2.3.2.1 Effect of varying protein concentration

The radioligand (250 pM) was incubated at 30°C for 2 h in the absence and presence of 10 µM R-PIA, with increasing amounts of the membrane protein (100 - 300 µg/tube), and the reaction terminated after 2 h by filtration.

2.3.2.2 Effect of temperature

The membrane protein (210 µg/tube) was incubated with 170 pM radioligand for 2 h, in the absence and presence of 10 µM R-PIA, at various temperatures ranging between 20 and 45°C.

2.3.2.3 Effect of pH

The membrane protein (100 µg/tube) was incubated with 330 pM radioligand at 30°C for 2 h, in the absence and presence of 10 µM R-PIA, and with Tris buffer of pH ranging between 7.0 and 9.0.

2.3.2.4 Kinetics analysis

The assay volume, including the amount of protein and radioligand, was scaled up several-fold in order to determine the rate of specific association of the radioligand with its receptor and the dissociation rate of the specifically bound radioligand from the receptor.

2.3.2.4.1 Association kinetics

The membrane suspension was added to the incubation mixture (30°C) containing either 140 or 480 pM radioligand at time zero. The rate of association of the radioligand was followed by withdrawing 450 µl of the incubation and filtering it as above, at various time intervals up to at least 2 h. The nonspecific binding component was determined simultaneously in the presence of 10 µM R-PIA using separate incubations. Similar assays were performed using 140 pM radioligand at 20°C to establish the time of equilibrium.

2.3.2.4.2 Dissociation kinetics

The radioligand (480 pM) was incubated with the membrane suspension at 30°C in the presence and absence of 10 μM R-PIA and allowed to equilibrate for 2 h. At 122 min, either R-PIA (10 μM) or a mixture of R-PIA and Gpp(NH)p (10 and 100 μM, respectively) was added to the incubation mixtures. The rate of dissociation of the radioligand was followed with time by withdrawing 450 μl from each set of incubations and filtering as above, at various time intervals.

2.3.2.5 Saturation analysis

Since the K_D of [^{125}I]R-IHPIA binding is in the nM range, unlabelled-IHPIA was used to dilute the specific activity of the radioligand. The radioligand (12.4 pmols) was dried under vacuum and the residue diluted about 35-fold with unlabelled-R-IHPIA to a specific activity of 56.7 Ci/mmole. Various quantities of this radioligand solution were incubated with a fixed amount of the membrane protein (100 μg/tube) at 30°C for 2 h, both in the absence and presence of 10 μM R-PIA.

2.3.2.6 Displacement analysis

The membranes (120 - 240 μg/tube) were incubated with 170 pM radioligand for 2 h, with increasing concentrations of the inhibitor, in a standard binding assay. In each assay, 10 μM R-PIA was used to define the nonspecific binding. The displacement curves were generated using 4 - 6 different concentrations of various inhibitors. In some assays, a single concentration (100 μM, final) of various

inhibitors was used.

2.4 Preparation of membranes from longitudinal muscle of rabbit small intestine

2.4.1 Tissue preparations

New Zealand White rabbits of both sexes weighing between 2.5 and 4 kg were killed by exsanguination. The small intestine extending from about 5 cm below the stomach to about 5 cm above the large intestine was removed. It was immersed in and gently flushed out with freshly prepared and aerated Krebs buffer of composition: 116 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 22 mM $NaHCO_3$ and 11 mM D-glucose, maintained at pH 7.4 by continuously gassing with 95% O_2/5% CO_2 at 37°C. The intestine was cut into small segments (5 - 8 cm) and maintained aerated throughout.

2.4.2 Fractionation of the longitudinal muscle

Each tissue segment was mounted individually on a glass pipette and the mesentery along with its underlying layer of longitudinal muscle was gently removed. Using a scalpel, a fine, superficial longitudinal slit was made along the entire length of the tissue segment. The longitudinal-muscle layer was then pulled off the entire length of the tissue segment using wet cotton tips and a pair of forceps, and kept aerated in Krebs solution.

2.4.3 Preparation of a 114,000 xg membrane pellet

The membranes were prepared using the general method for smooth-muscle membrane preparation described by Kidwai (1975). After the Krebs buffer had been drained, the longitudinal-muscle strips were cut into small pieces on an ice-cold petri dish. The tissue was now homogenized in 5 volumes of a buffer of 0.25 M sucrose, 50 mM Tris HCl (pH 7.4), and 2 mM $MgCl_2$ using a polytron homogenizer at the setting of six, with four pulses each of 5 sec duration. Between each pulse, the tube containing the homogenate was maintained on ice. The homogenate was then centrifuged at 1,000 xg in a Sorval SW centrifuge for 10 min (2°C), when the pellet (P_1) was discarded and the supernatant recentrifuged at 10,000 xg for 10 min (2°C) in the same centrifuge. The pellet (P_2) obtained from this run was also discarded and the supernatant recentrifuged at 114,000 xg for 40 min (2°C) in a Beckman LS-40 ultracentrifuge. The resulting pellet (P_3) was washed once in the same buffer but without the sucrose at 114,000 xg for 40 min. The final washed pellet was redispersed in the sucrose-free buffer to a concentration of 2 - 4 mg protein/ml, and the solution divided into aliquots, stored frozen under liquid nitrogen and used in binding assays within two weeks. Protein was determined by the method of Lowry et al. (1951). In our early experiments with the smooth-muscle membranes, the pellets P_1 and P_2 were ~~not~~ discarded but each was washed once in sucrose- free buffer, redispersed in the same buffer, divided into aliquots and stored frozen under liquid nitrogen.

2.4.4 Assay of adenylate cyclase as the longitudinal-muscle membrane marker

Adenylate cyclase activity was measured according to the method of Baer (1975). The reaction mixture contained 0.1 mM α-^{32}P-ATP, 50 mM Tris HCL (pH 7.4), 5 mM $MgCl_2$, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.04% bovine serum albumin, and 9 µg protein from the homogenate or from each pellet P_1, P_2, or P_3, in a total volume of 50 µl. Chromatographic separation of the formed ^{32}P-cAMP and subsequent radioactive counting was done as described under section 2.2.2.

2.5 Radioligand binding assays with the longitudinal muscle membranes of rabbit small intestine

2.5.1 Standard assay protocol

Binding assays were performed using three different radioligands ([^{125}I]-labelled-R-IHPIA, [^{3}H]-labelled R-PIA, and NECA). The standard protocol of binding assays with each radioligand was similar, and was as follows.

2.5.1.1 Filtration-binding assay

The membranes were thawed and incubated with the radioligand and other drugs as indicated, in a final volume of 0.5 ml, containing 50 mM Tris HCl (pH 7.4) and 2 mM $MgCl_2$. The binding reactions were initiated by adding membranes and terminated by removing 450 µl of the incubations and filtering through wetted, precooled glass-fibre

ml of ice cold buffer. When radioiodine-labelled ligand v
binding assays, the filter papers were counted in a Gamma
described under section 2.3.2. When tritium-labelled li
used, the filter papers were transferred to scintilla
containing 10 ml of the scintillation fluid (ACS, Amersha
radioactivity counted in a Beckman LS-7500 Beta counter
precalibrated for quench correction using a set of trit
standards (Amersham). The nonspecific binding was defi
binding displaced by 100 μM R-PIA ([^{125}I]R-IHPIA), 50(
([^{3}H]R-PIA), and 500 μM NECA ([^{3}H]NECA). Irrespective of
specificity, the radioligand binding displaceable in the
an excess of the cold ligand (R-PIA or NECA, as the case
referred to as specific binding.

2.5.1.2 Centrifugation-binding assay

The membrane suspension (the pellet P_3) was centrif
Eppendorff microcentrifuge at about ~12,800 xg for 2 min.
was resuspended in sucrose-free buffer. This membrane sus
used to initiate binding reactions in a standard incubati
and the reaction was terminated by centrifugation in a micr
as above. The supernatant did not contain any detectabl
protein. No correction for the trapped space in the pelle
When radioiodinated ligand was used, the pellets were cc
Gamma counter as described under section 2.3.2. Whe
labelled ligands were used, the pellets were solubilized by

overnight with 100 µl of NCS solubilizer (Amersham) at 45°C. The solubilisate's pH was neutralized with glacial acetic acid before it was transferred to scintillation vials containing 10 ml of ACS scintillation fluid, and radioactivity was counted as described under section 2.5.1.1.

2.5.2 [^{125}I]R-IHPIA binding assays with the longitudinal muscle membranes

The membrane protein (80 - 100 µg) was incubated with the radioligand in a standard binding assay at 20°C for 10 min (with some exceptions, which are indicated) and the nonspecific binding was defined as the binding displaced by 100 µM R-PIA.

2.5.2.1 Effect of pretreatment of the membranes with adenosine deaminase

The membranes were pretreated with 1 - 4 International Units adenosine deaminase per ml of protein suspension at 37°C for 30 min and used with 190 pM radioligand in a standard binding assay at 20°C. The reaction was terminated after 10 min by filtration.

2.5.2.2 Effect of varying protein concentration

The radioligand (400 pM) was incubated with increasing amounts of membrane protein (62 - 256 µg/tube) at 20°C for 10 min, in the absence and presence of 100 µM R-PIA. The reaction was terminated by filtration.

2.5.2.3 Effect of temperature

The membrane protein (80 µg/tube) was incubated with 190 pM radioligand for 60 min, in the absence and presence of 100 µM R-PIA, at various temperatures ranging between 10 and 37°C. The reaction was terminated by filtration.

2.5.2.4 Ion concentration effect

The membrane protein (100 µg/tube) was incubated at 20°C, with 330 pM [^{125}I]R-IHPIA and increasing concentrations of Na^{+}, Mg^{2+}, and Ca^{2+} in a standard binding assay, and the reaction terminated by filtration at the end of 10 min.

2.5.2.5 Kinetic analysis

The assay volume, including amounts of protein and radioligand, was scaled up several fold. The membrane protein (84 µg/500 µl) was added at time zero to the incubations containing 190 pM radioligand at 20°C, and the rate of association was followed by withdrawing 450 µl of the incubation and filtering it as previously described at various time intervals. The nonspecific binding component was determined using 100 µM R-PIA in separate incubations. In order to induce dissociation, R-PIA was added to the incubation to a final concentration of 100 µM and the rate of dissociation followed with time as described above, for up to 10 min.

2.5.2.6 Displacement analysis

The membrane protein (100 µg/tube) was incubated with 252 pM radioligand and a single concentration (100 µM) of each inhibitor at 20°C for 10 min before the reaction was terminated by filtration. No displacement curves were generated.

2.5.3 [^{3}H]R-PIA binding assays with the longitudinal muscle membranes

The membrane protein (90 - 160 µg) was incubated with the radioligand in a standard binding assay at either 2°C or 20°C for 10 min and the nonspecific binding was defined as the binding displaced by 500 µM R-PIA.

2.5.3.1 Effect of varying the protein concentration

The radioligand (20 nM) was incubated with increasing amounts of membrane protein (62 - 326 µg/tube) at 20°C for 10 min, in the absence and presence of 500 µM R-PIA. The reaction was terminated by filtration.

2.5.3.2 Effect of temperature

The membrane protein (160 µg/tube) was incubated with 20 nM radioligand for 10 min, in the absence and presence of 500 µM R-PIA, at various temperatures ranging between 2 and 37°C. The reaction was terminated by filtration.

2.5.3.3 Effect of pH

The membrane protein (112 µg/tube) was incubated with 20 nM

radioligand at 20°C for 10 min in the absence and presence of 500 μM R-PIA, and with Tris buffer of pH ranging between 7 and 9. The reaction was terminated by filtration.

2.5.3.4 Kinetic analysis

The association and dissociation kinetic reactions were performed similarly to those described under section 2.5.2.6 for [^{125}I]R-IHPIA binding. The kinetics was studied using 140 μg protein/500 μl and 20 nM radioligand at 2°C. The nonspecific binding was determined using separate incubations in the presence of 500 μM R-PIA. The dissociation was induced with R-PIA at a final incubation concentration of 100 μM.

2.5.3.5 Displacement analysis

The membrane protein (132 μg/tube) was incubated with 30 nM radioligand and a single concentration (100 μM, final) of the inhibitor at 20°C for 10 min before the reaction was terminated by filtration. No displacement curves were generated.

2.5.4 [^{3}H]NECA binding assays with the longitudinal muscle membranes

The membrane protein (100-160 μg/tube) was incubated with the radioligand in a standard binding assay at 2°C for 20 min and the nonspecific binding was defined as the binding displaced by 500 μM NECA.

2.5.4.1 Effect of pretreatment of the membranes with adenosine deaminase

The membranes were pretreated with 0.1 - 5 U adenosine deaminase per ml of protein suspension (96 µg/tube) at 37°C for 30 min and used with 20 nM radioligand in a standard binding assay at 2°C. The reaction was terminated after 20 min by filtration.

2.5.4.2 Effect of varying protein concentration

The radioligand (10 nM) was incubated with increasing amounts of the membrane protein at 2°C for 20 min, in the absence and presence of 500 µM NECA. The reaction was terminated by filtration.

2.5.4.3 Effect of temperature

The membrane protein (160 µg/tube) was incubated with 30 nM radioligand for 20 min in the presence and absence of 500 µM NECA, at various temperatures ranging between 2°C and 37°C. The reaction was terminated by filtration.

2.5.4.4 Effect of pH

The membrane protein (200 µg/tube) was incubated with 20 nM radioligand at 2°C for 20 min in the absence and presence of 500 µM NECA and with the Tris buffer (pH ranging between 7 and 9). The reaction was terminated by filtration.

2.5.4.5 Kinetic analysis

The association and the dissociation kinetic reactions were performed similarly to those described under section 2.5.2.6 for [^{125}I]R-IHPIA binding. The kinetics of the reaction was studied with 20 nM radioligand at 2°C. Nonspecific binding was determined using separate incubations in the presence of 500 μM NECA. Dissociation was induced with NECA at a final incubation concentration of 100 μM.

2.5.4.6 Saturation analysis

Since the K_D of [^{3}H]NECA binding is in the μM range, unlabelled NECA was used to dilute the specific activity of the radioligand. Radioligand (1.6 nmols) was diluted 10-fold with unlabelled NECA to a specific activity of 2.2 Ci/mmole. Various quantities of this radioligand solution (to achieve final incubation concentration of 0.1 - 2 μM) were incubated with a fixed amount of the membrane protein (132 μg/tube) at 2°C for 20 min, in the absence and presence of 500 μM NECA before the binding reaction was terminated by filtration. The lowest concentration point corresponding to 0.02 μM concentration was used as the original specific activity of 22 Ci/mmole.

2.5.4.7 Displacement analysis

The radioligand (20 nM) and membrane protein (100 - 140 μg/tube) were incubated at 2°C for 20 min with different concentrations of various inhibitors in standard binding assays. In each assay, 500 μM NECA was used to define the nonspecific binding. The displacement curves were generated using 4 - 7 different concentrations of the

various inhibitors. Compounds that were weak inhibitors of binding were used at a single concentration (100 μM, final).

2.6 Smooth muscle contractility studies

Portions of rat small intestine (jejunum, 2-cm length) were excised as described in sec. 2.4.1, and mounted longitudinally in [illegible] organ baths, at 37°C, under an initial tension of 1 g; the isometric force was measured by means of Grass FT03C force transducers. Following a 30 - 60 min period, increasing concentrations of the nucleosides were added to the baths noncumulatively. The concentration-response curves for a given nucleoside were generated in paired strips in the absence and presence of 100 μM 8-p-sulfophenyl-theophylline (SPT) which was added 15 min before nucleoside additions.

2.7 Adenylate cyclase activity in murine neuroblastoma cell membranes

2.7.1 Preparation of a 10,000 xg membrane pellet

The cells were grown in monolayer using petri dishes. Gibco F-16 containing 10% fetal calf serum and 7.5% gentamycin sulphate was used as the growth medium. The cells were scraped off the petri dishes using a rubber policeman and the cell suspension centrifuged in an international clinical centrifuge at maximum speed for 20 min. The pelleted cells were washed three times in a buffer containing 0.25 M

sucrose, 10 mM Tris HCl (pH 7.5) and 1 mM $MgCl_2$. The packed cells were homogenized in 10 volumes of the same buffer using a Potter Elvehjem homogenizer with teflon pestle (15 strokes) and the homogenate centrifuged at 10,000 xg for 20 min at 2°C, in a Sorval SV centrifuge. The pellet was washed three times and resuspended in the same buffer. Protein was determined by the method of Lowry et al. (1951).

2.7.2 Assay of the stimulatory adenylate cyclase activity

Adenylate cyclase activity was measured according to the method of Baer (1975) using α-^{32}P-ATP as the substrate and PEI TLC to separate the formed radioactive cAMP. The assay protocol was same as described under section 2.5.3 for adenylate cyclase from rabbit intestinal-muscle. 16 - 20 µg protein was used in a total volume of 50 µl.

2.8 Data analysis

2.8.1 Ligand binding data

The amount of the radioligand bound (cpm) was converted into fmols bound per mg of protein using as parameters radioligand specific activity, counter efficiency and the incubation protein concentration, on a computer programme. In the case of binding assays using tritium labelled ligands, tritium quench standards were used to calculate the counting efficiency. The procedure may be summarized as follows. Using the dpm present in the quench standards (corrected for radio-

active decay), the ratio of cpm to dpm (counting efficiency) was plotted on the ordinate against the corresponding H-numbers on the abscissa. The slope of the line (-m) and its intercept on the ordinate (c) were used along with the values for the radioligand specific activity and the protein concentration in the incubation in a computer-programmed equation, to obtain fmols or pmols of the bound radioligand per mg of protein (Appendix III). For the binding assays utilizing rabbit small intestinal membranes, because 20% of the total protein in the incubation passed through G/F filters, the pmols bound were calculated as the amount of protein retained on the filter paper that is, 80% of the incubation protein amount.

2.8.1.1 Estimation of the binding constants

The data from various receptor binding assays were expressed as parameters such as dissociation constant, rate constants and the receptor densities.

2.8.1.1.1 Analysis of saturation data

Since the relationship between ligand concentration and the receptor occupancy is hyperbolic, the values for dissociation constant (K_D) and the receptor density (B_{max}) were obtained by a non-linear curve fitting approach, based on ligand binding models and equations developed by Feldman (1972), using an iterration programme (GIP) on a Hewlett-Packard computer HP9825. The saturation binding data were also transformed graphically to give a linear relationship on two different plots: (1) Scatchard (1949) using the equation $B/F = -B/K_D$

+ B_{max}/K_D (where B represents fmols/mg bound specifically at various radioligand concentrations and F is the incubation concentration of the free radioligand), and (2) Hill (1910) using the equation $\log[B/(B_{max}-B)] = n^H.\log F$, where B, B_{max} and F have the same connotation as above, and n^H represents the Hill coefficient given by the slope of the linear plot.

2.8.1.1.2 Analysis of kinetic data

As the concentrations of the radioligand and receptor in the incubations (rat brain membranes) were similar, the specific binding data from the forward reaction of [^{125}I]R-IHPIA with brain adenosine receptors was linearized using the second order equation (Weiland and Molinoff, 1981):

$$k_1.t = [\ln[Be(L-B.Be/R_T)/(L.(Be-B)]]/(L.R_T/Be-Be) \quad (1)$$

where B is the incubation concentration of the specifically bound radioligand at different time points (t), Be is the concentration of the same at equilibrium, L is the initial concentration of free radioligand which was assumed to be equal to the radioligand concentration of the total incubation and R_T is the concentration of the receptor in the incubation which was determined using the B_{max} value obtained from saturation analysis. The slope of the plot provided the estimate for the second-order forward-rate constant (k_1). The forward rate constant for [^{3}H]NECA binding with NECA- displaceable binding sites in smooth muscle membranes was determined using the pseudo first order equation since the receptor concentration in the incubation is much lower than the K_D. Equation 1 then reduces to:

$$k_1.t = Ln(Be/Be-B)Be/L.R_T \quad (2)$$

The slope of the plot is equal to $k_1.L.R_T/Be$; from which k_1 can be calculated. R_T was calculated from saturation analysis of the binding (Fig. 27).

The specific binding data obtained from the dissociation reaction was linearized using the equation $k_2.t = \ln (B/Be)$, where Be represents fmols/mg protein bound per incubation at equilibrium (just before addition of the unlabelled ligand) and B represents the same at different time points (t) after dissociation had begun. The first order rate constant for the dissociation reaction (k_2) was calculated from a similar plot but using the specific binding measured 3 min after the addition of the unlabelled ligand as Be. The slope of the plot provided the value for k_2.

2.8.1.1.3 Analysis of displacement data

Concentration response curves for each individual inhibitor were drawn. IC_{50} (concentration of the inhibitor required for 50% inhibition of the specific binding) and Hill coefficient (n^H) values were estimated from Hill plots using the equation $\log (P/100-P) = n^H.\log D$, where P represents percentage inhibition of specific binding at various concentrations (D) of the inhibitor. The IC_{50} values obtained as above from [^{3}H]NECA binding data with small-intestinal membranes, were transformed into Ki (inhibitory constant) using the equation provided by Cheng and Prusoff (1973); $Ki = IC_{50}/(1 + F/K_D)$, where F is the concentration of the free radioligand (taken as equal to the total radioligand concentration) and K_D was obtained

from the saturation analysis. For displacement binding data using rat brain membranes, the IC_{50} values were transformed into Ki using modifications of the equation of Cheng and Prusoff made by Linden (1982), to account for the higher concentration of receptors in the incubations (close to K_D). The equations used are:

$$F^2 + F.(R_T-L+K_D) - K_D.L = 0 \quad (3)$$

$$I_f = IC_{50} - R_T + (R_T/2).[F/(K_D+F)+K_D/(K_D+F+R_T/2)] \quad (4)$$

$$Ki = I_f/[1+F/K_D+R_T/K_D((K_D+F/2)/K_D+F)] \quad (5)$$

where L is the [redacted]ncentration of the radioligand in the absence of an inhibitor, I_f is the free concentration of the inhibitor, and F, K_D, and R_T have the same connotations as before.

2.8.2 Analysis of adenylate cyclase data

The ratio of cpm in the ATP/deoxyATP to cyclic AMP/ deoxycyclic AMP spots provided a measure of the percentage conversion of the nucleotide to the cyclic nucleotide. The values for the percentage conversion were transformed into pmols of cyclic nucleotide formed per mg per min on a computer using incubation protein concentration and time as parameters. To estimate ED_{50} values with 95% confidence limits and test for statistical significance for paired data, the same procedure as described below under section 2.8.3 for contractility studies was used.

2.8.3 Analysis of contractility data

Semi-logarithmic concentration-response curves for each individual strip were drawn, log ED_{50} values were interpolated, and

geometric means of ED_{50} values (with 95% confidence limits) were calculated. Values of log ED_{50} obtained in the absence and presence of 100 μM SPT were compared by Student's t-test for paired data (Hill, 1971), and the differences were considered significant when $p<0.05$.

2.8.4 Presentation of the data

Most data are presented as triplicates of a single determination unless stated otherwise in the various figure legends. The ranges of standard deviation or standard error of the mean (S.E.M.) also appear in the figure legends. The brain membranes were prepared using at least 3 rats each time and the intestinal membranes using between 2 - 4 (usually 3) rabbits each time.

2.9 Materials

2.9.1 Radiochemicals

Carrier-free Na-^{125}I (2000 Ci/mmol) was obtained from Radiopharmaceutical Centre, University of Alberta, [^{3}H]R-PIA (42 Ci/mmol) and [^{3}H]NECA (21/22 Ci/mmol) from Amersham Corporation, and α-^{32}P-ATP (25 Ci/mmol) and α-^{32}P-deoxy-ATP (25 Ci/mmol) from ICN (Irvine, CA, USA).

2.9.2 Chemicals, drugs, and other materials

Silica-gel TLC plates were obtained from Merck AG (Darmstadt, F.R.G.); PEI TLC plates from Macherey-Nagel, Brinkman Instruments, Canada; X-Ray films from X-Omat AR, and PPO and POPOP from Eastman

Kodak, Rochester, NY, USA. The following were purchased from Sigma Co. (St Louis, MO, USA): adenosine deaminase (Type III), creatine phosphokinase, trypsin, guanine, inosine, 5'-deoxy-5'-methylthioadenosine, 2-chloroadenosine, 5'-deoxy-5'-chloroadenosine, L and D isomers of S-adenosylhomocysteine, dipyridamole, and dithiothrieotol. NECA, R-PIA, CHA, 2'-deoxyadenosine, 3'-deoxyadenosine, GTP, Gpp(NH)p, creatine phosphate, cyclic AMP, deoxycyclic AMP, AMP, deoxyAMP, ATP, deoxyATP, and inosine were purchased from Boehringer Mannheim (Mannheim, F.R.G.); S-PIA and 8-p-sulfophenyltheophylline from RBI Chemicals (Wayland, MA, USA); 2':5'-dideoxyadenosine from P.L. Biochemicals (Milwaukee, WI, USA); 8-phenyltheophylline from Calbiochem-Behringer (La Jolla, CA, USA); and IBMX from Aldrich Chemical Co. (Milwaukee, WI, USA).

NBMPR was kindly supplied by Dr. A.R.P. Paterson (McEachern Laboratory, University of Alberta, Edmonton), L- and D-enantiomers of p-hydroxyamphetamine by Mr. H.A. Shepard (SKF Canada Ltd., Mississauga, ON), and R/S-IHPIA by Dr. Weiman (Boehringer Mannheim, Mannheim, F.R.G.). RO-20-1724 was received from Hoffman La Roche (Nutley, NJ, USA).

All other chemicals used were from standard sources.

2.9.3. Preparation of drug solutions

All nucleosides and other water-insoluble drugs were dissolved in DMSO, unless otherwise stated, to a stock concentration of 50 mM (10 mM for Dipyridamole and 1 mM for RO-20-1724), such that the maximum DMSO concentration per incubation ranged between 0.2 - 2% in various

assays of ligand binding and adenylate cyclase. The maximal ethanol concentration in cerebellar adenylate cyclase assays was 2%. Theophylline, 8-p-sulfophenyltheophylline, cyclic AMP and deoxycyclic AMP were dissolved in buffer. Nitrendipine was dissolved in ethanol at a stock concentration of 10 mM and care was taken not to expose the solutions of nitrendipine and verapamil to light. All drug solutions were stored either at 4°C or -20°C. Buffers of various pH for studies of pH effect were made using a stock Tris buffer of pH 9.0 and titrating it against HCl. The pH of all buffers was adjusted at ambient temperature.

All tissue contractility studies were performed by Mr. Richard Vriend from Dr. H.P. Baer's laboratory.

3. RESULTS

3. RESULTS

3.1 Identification of the product of radioiodination of R-HPIA

The radioiodination-product of HPIA showed a single radioactive spot on autoradiograms, using both silica gel and PEI TLC systems. This radioiodination product co-chromatographed with the unlabelled R/S-IHPIA thus validating the identity of the radioligand (Fig. 2). Thus in our radioiodination reaction there was no detectable diiodo-derivative formed. Although the incorporation of ^{125}I was >90%, the final yield of [^{125}I]R-IHPIA was only about 32%. This is due to low recovery of the radioligand from PEI plates.

3.2 Biological activity of R-IHPIA

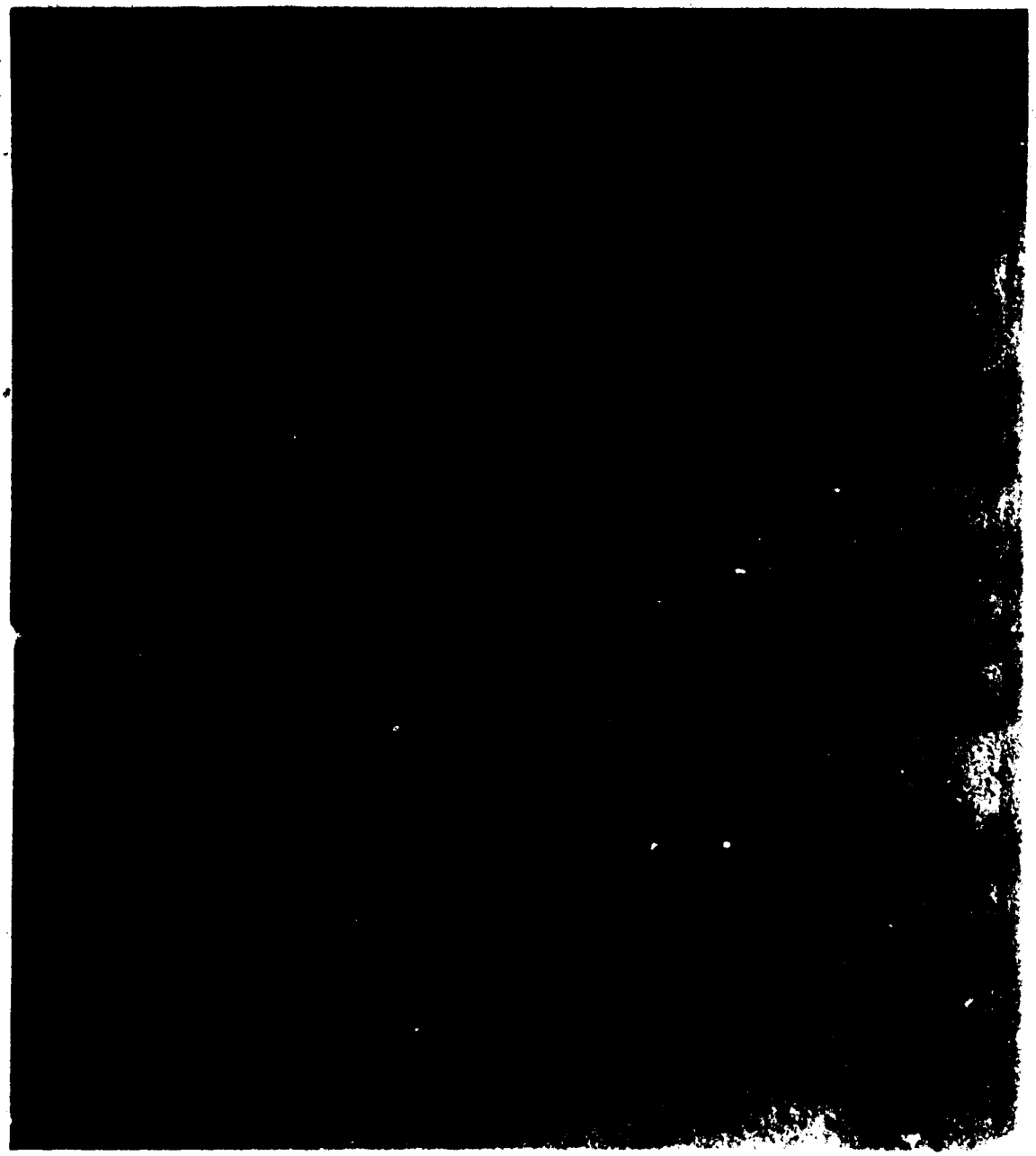
The basal adenylate cyclase activity in cerebellum (107 - 114 pmols/mg/min) was inhibited by a low concentration of GTPγS (nM range, Fig. 3), suggesting that the Ni unit in the membrane preparation is intact and functional. In a preliminary assay, both R-PIA and R-IHPIA inhibited the basal enzyme activity in a dose-dependent fashion (Fig. 4). The maximal inhibition obtained was 39% (R-PIA) and 36% (R-IHPIA), each at 10 μM concentration. The concentration for half maximal inhibition was 35 nM for R-PIA and 100 nM for R-IHPIA.

3.3 Measurement of [^{125}I]R-IHPIA binding in brain membranes

3.3.1 Duration of and temperature stability of the radioligand

Radioligand (0.26 nM) was incubated under standard binding

Fig. 2. Identity of the radioactive product. Autoradiograph of the silica gel (panel 1) and PEI (panel 2) TLC developed as described in Materials and Methods. Lane A: HPIA; lane B: IHPIA; lane C: [^{125}I]R-IHPIA; lane D: N^6-[(3,5-diiodo-4-hydroxyphenyl)isopropyl]adenosine (I_2HPIA).



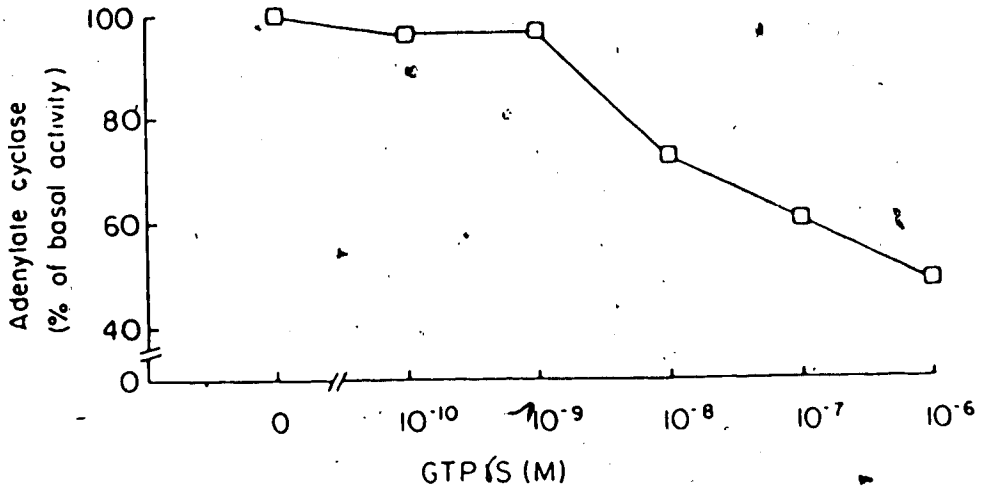


Fig. 3. Effect of GTPγS on the basal adenylate cyclase activity in rat cerebellar membranes. Adenylate cyclase activity was measured at 20°C for 20 min. Values are a mean of triplicate determinations with standard deviations at most points between 5 - 11%.

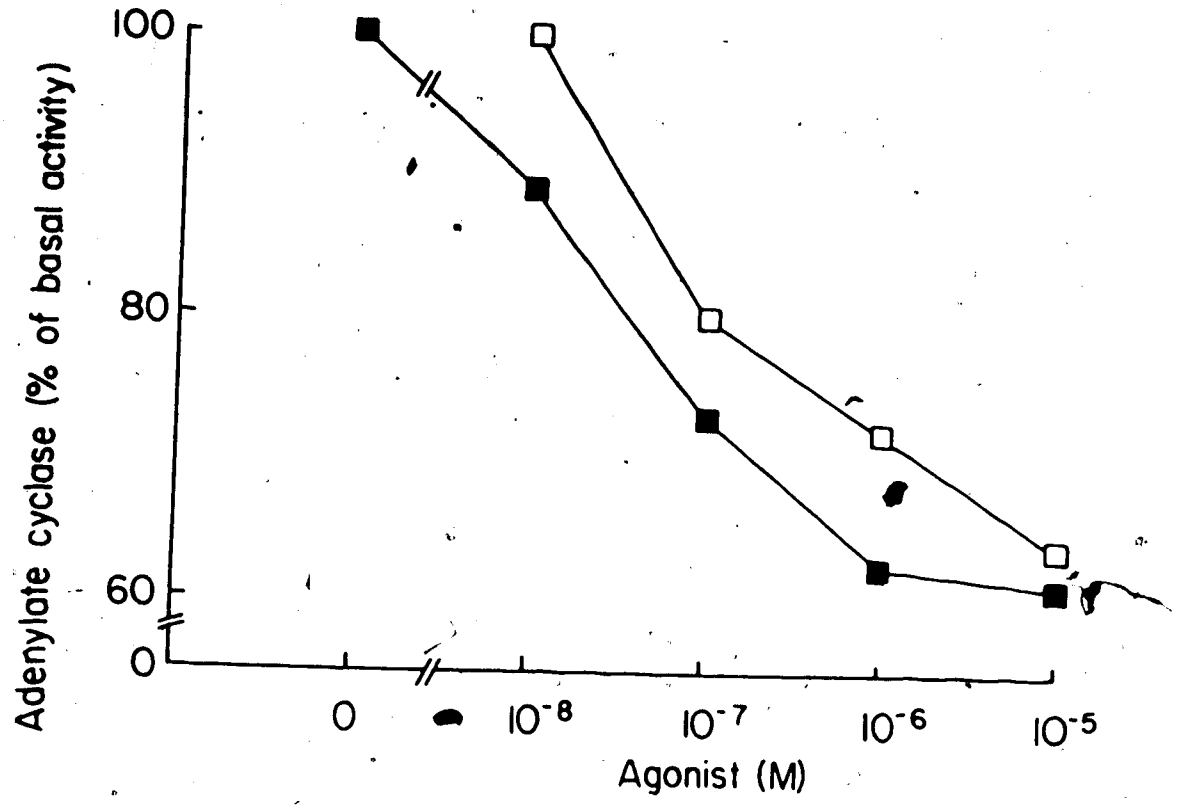


Fig. 4. Effect of R-PIA and R-IHPIA on basal adenylate cyclase activity in rat cerebellar membranes. Inhibition of adenylate cyclase activity by R-PIA (■) and R-IHPIA (□) was measured at 20°C for 20 min, in the presence of 10 μM GTP. Values are a mean of triplicate determinations with standard deviations at most points between 6 - 14%.

conditions in the presence and absence of membranes (200 µg) at temperatures of 30°C and 37°C. Aliquots (3 µl) were removed every 10 min from the incubation mixtures up to 2 h, spotted on PEI TLC and developed using 0.25 M LiCl. At both temperatures, a single radioactive spot was observed on the autoradiogram of the developed TLC sheet for each time point, which co-chromatographed with the radioligand. This indicates that the radioligand is stable under standard incubation conditions and at these temperatures up to at least 2 h.

3.3.2 Identification of bound radioactivity

Rat brain membranes (600 µg protein) were incubated with 300 pM radioligand under standard assay conditions and filtered as described under section 2.1.3.1. The filter paper was rapidly transferred to a glass tube containing 1.5 ml of ethylacetate and let stand for 10 min. After removal of the filter paper, the ethylacetate extract was concentrated under vacuum and part of the concentrate was applied to PEI TLC for chromatographic visualization (autoradiography); the remaining material was reused in a binding assay under standard conditions. The ethylacetate extract of the filter paper-bound radioactivity, co-chromatographed with the radioligand. Also, the extracted radioligand showed specific binding identical to that obtained from the stock ligand preparation with the same amount of radioactivity.

3.3.3 Effect of washing

The specific binding was similar when the filter papers were

washed either 2 or 3 times. If the filter papers were given a single 5-ml wash, the nonspecific binding was much higher and the specific binding slightly lower than that after 2 or 3 washes. Two volumes of 5 ml each were used to wash each filter paper in all subsequent assays (data not shown).

3.3.4 [redacted]arying the protein concentration

Both the total and nonspecific binding was linear up to 300 µg protein per incubation (0.6 mg/ml, Fig. 5). Therefore, 100 - 240 µg membrane protein was used per incubation in various binding assays.

3.3.5 Effect of temperature

Fig. 6 shows the temperature dependence of the binding reaction. Although the nonspecific binding changed only marginally as temperature was increased from 20 to 45°C, the observed specific binding was maximum between 20 - 30°C. On the basis of these preliminary observations a temperature of 30°C was chosen for all subsequent studies.

3.3.6 Effect of pH

Nonspecific binding decreased progressively as the pH was increased from 7 to 9 (Fig. 7A). Specific binding was maximal between pH 7 and 8 and decreased sharply as the pH was increased from 8 to 9 (Fig. 7A). A pH of 7.4 was chosen for all subsequent assays. Fig. 7B shows that the specific binding of [^{3}H]R-PIA does not change appreciably between the pH of 7 and 9.

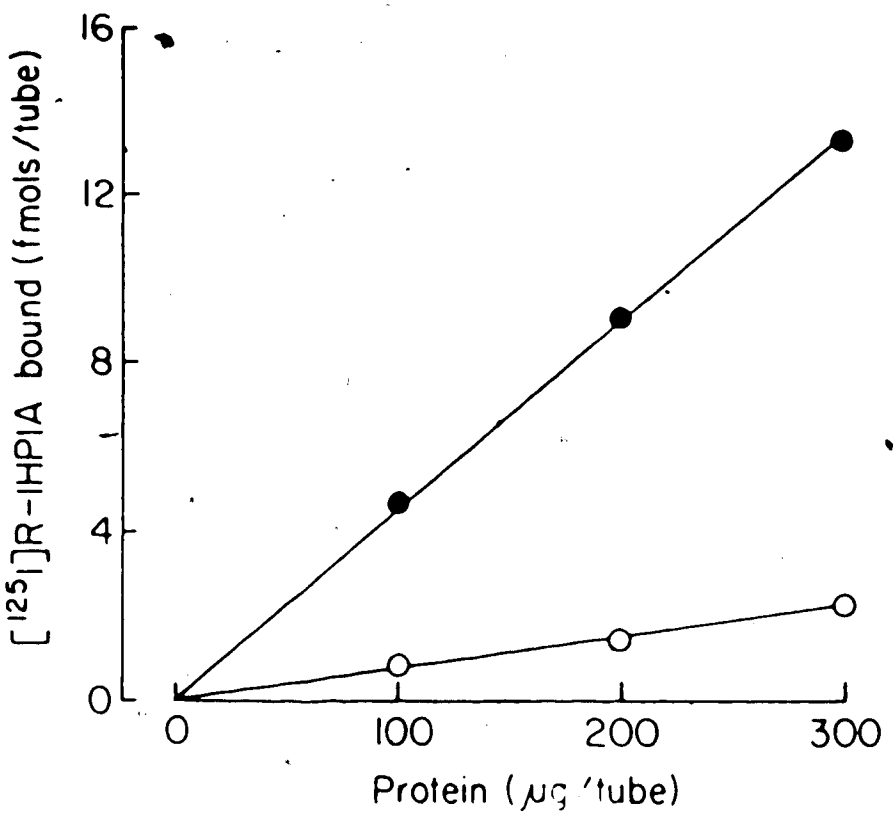


Fig. 5. Effect of variation in protein concentration on [^{125}I]R-IHPIA binding to rat brain membranes. [^{125}I]R-IHPIA (250 pM) binding was measured in the absence (●) and presence (○) of 10 µM R-PIA at 30°C for 2 h. Values are a mean of triplicate determinations with standard deviations of less than 7%.

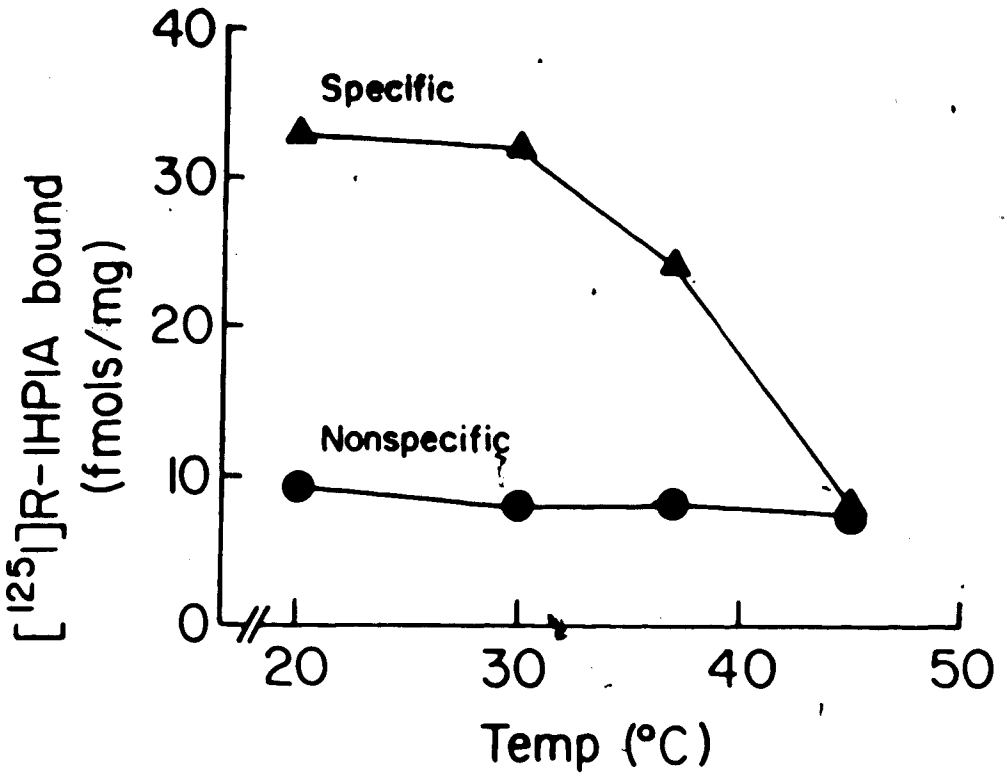
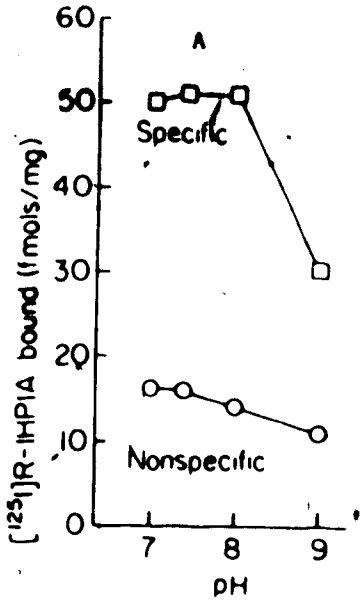
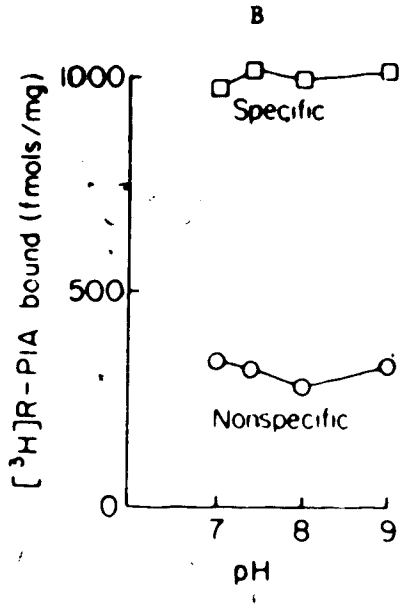


Fig. 6. Effect of temperature variation on [^{125}I]R-IHPIA binding to rat brain membranes. [^{125}I]R-IHPIA (170 pM) binding was measured for 2 h at various temperatures as shown on the abscissa. Values are a mean of triplicate determinations with standard deviations of less than 11%.

Fig. 7. Effect of pH on (A) [^{125}I]R-IHPIA and (B) [^{3}H]R-PIA binding to rat brain membranes. A. [^{125}I]R-IHPIA (330 pM) binding was measured at 30°C for 2 h and at various values of pH in the incubation buffer, as shown on the abscissa. Similar results were obtained in another experiment. B. [^{3}H]R-PIA binding was measured by the method of Schwabe and Trost (1980), at 30°C for 20 min using 20 nM radioligand and 110 µg protein per incubation. All values are a mean of triplicate determinations with standard deviations of less than 8%.


A
60
50
40
30
20
10
0
Specific
Nonspecific
[125I]R-IHPIA bound (fmols/mg)
7
8
9
pH


B
1000
500
0
Specific
Nonspecific
[3H]R-PIA bound (fmols/mg)
7
8
9
pH

3.3.7 Determination of binding constants

3.3.7.1 Kinetic analysis

An incubation time of 2 h was required at both 140 and 480 pM concentrations of radioligand, for the system to attain equilibrium at 30°C (Fig. 8). The binding reaction at 20°C also equilibrated at in 2 h. The second-order rate constant for the association reaction (k_1) as obtained from the slope of the plot (shown in the inset to Fig. 8) was 7.5×10^6 M^{-1} min^{-1}. Upon addition of a high concentration of the unlabelled ligand R-PIA, the bound radioligand at equilibrium dissociated rather slowly with a $t_{1/2}$ ($=\ln 2/k_2$) of 41.8 min. Addition of Gpp(NH)p along with R-PIA accelerated the rate of dissociation (Fig. 9). The nonspecific binding, both in the absence and presence of Gpp(NH)p, did not change appreciably up to at least the first 2 h of dissociation.

Log transformation of the specific binding data revealed a biphasic nature of dissociation both in the absence and presence of Gpp(NH)p. About 20% of the specifically bound radioligand (in the absence of Gpp(NH)p) dissociated within the first 3 min followed by a more gradual dissociation rate (Fig. 9, inset 1). The first order dissociation plot using the specific binding data (in the absence of Gpp(NH)p) up to the first 60 min of dissociation (Fig. 9, inset 2) provided the estimate for the dissociation rate constant, k_2 ($=0.018$ min^{-1}). Using these values of the rate constants k_1 and k_2, the dissociation constant ($K_D=k_2/k_1$) was estimated to be 2.4 nM.

Fig. 8. Kinetics of association of specific binding of [^{125}I]R-IHPIA to rat brain membranes [^{125}I]R-IHPIA, 480 pM (●) or 140 pM (○), was incubated with rat brain membranes at 30°C. After various times, as indicated, 450-µl aliquots were removed from the incubation and filtered (see section 2.3.2.4.1.). Nonspecific binding was determined in the presence of 10 µM R-PIA. Values are a mean of triplicate determinations with standard deviations of 4 - 14% at various points. Inset: The specific binding data using both concentrations of the radioligand were linearized using the second-order rate equation (1) (section 2.8.1.1.2). The total radioligand concentrations (480 and 140 pM) were taken as L. R_T (445 pM) was calculated using the B_{max} value from Fig. 10. B_e was measured as the specific binding (B) at the end of 2 h and B was calculated by substracting the average of nonspecific binding at various time points from the total binding at each time point. The common slope (r=0.97) of the plot from both radioligand concentrations, provided the estimate for the second-order rate constant, k_1=7.5 x 10^6 M^{-1} min^{-1}.

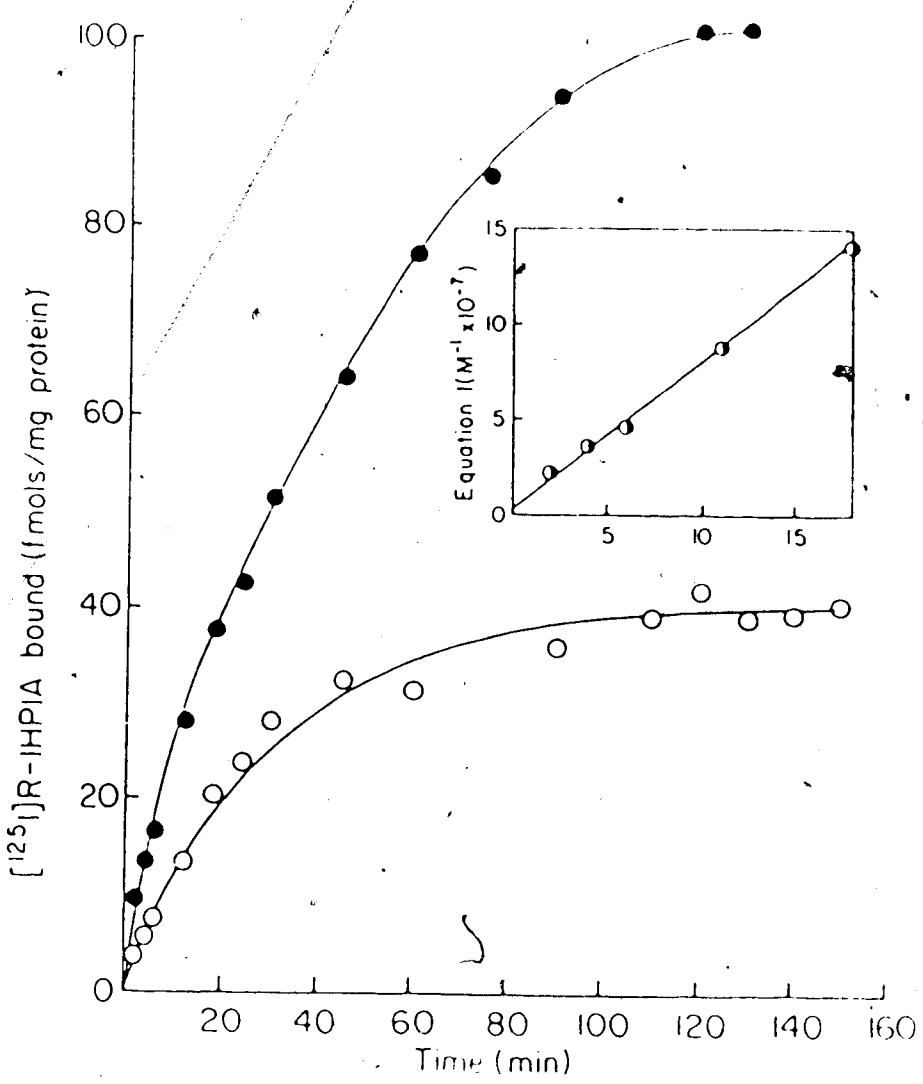
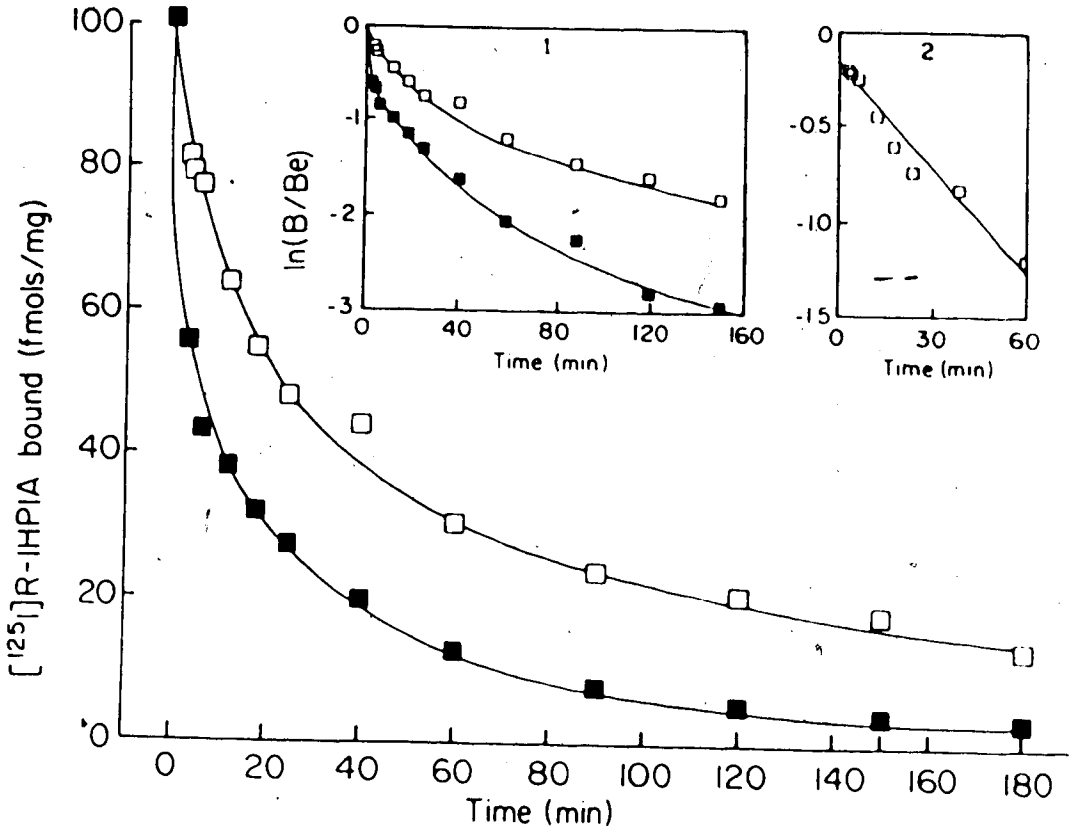

100
80
60
40
20
0
[125I]R-IHPIA bound (fmols/mg protein)
20
40
60
80
100
120
140
160
Time (min)
15
10
5
0
Equation 1/(M-1 x10-7)
5
10
15

Fig. 9. Kinetics of dissociation of specific binding of [^{125}I]R-IHPIA to rat brain membranes. [^{125}I]R-IHPIA (0.48 nM) was incubated with rat brain membranes at 30°C for 2 h. R-PIA (10 μM); □) or 10 μM R-PIA + 100 μM Gpp(NH)p (■) was rapidly added to the incubation mixture at the end of 2 hr. Aliquots (450 μl) were removed from the incubation at various times and filtered. Values are a mean of triplicate determinations with standard deviations of 3 - 12% at various points. Inset: first-order plots of the dissociation of [^{125}I]R-IHPIA from the binding sites. 1. B_e represents specific binding just before addition of R-PIA (time zero) and B represents the same at various times after addition of R-PIA. 2. Same as 1 but up to first 60 min of dissociation in the absence of Gpp(NH)p. The slope of this plot (r=0.97) provided the estimate for the first order rate constant, k_2 = 0.018 min^{-1}.


[125I]R-IHPIA bound (fmols/mg)
Time (min)
ln(B/Be)
1
2
Time (min)
Time (min)

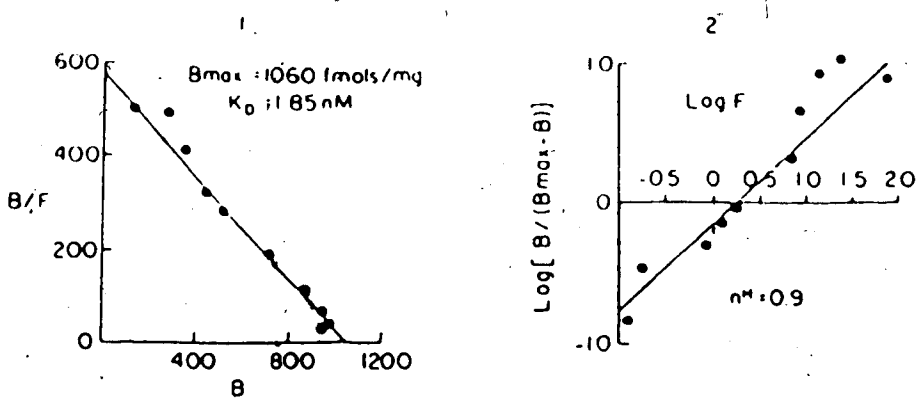
3.3.7.2 Saturation analysis

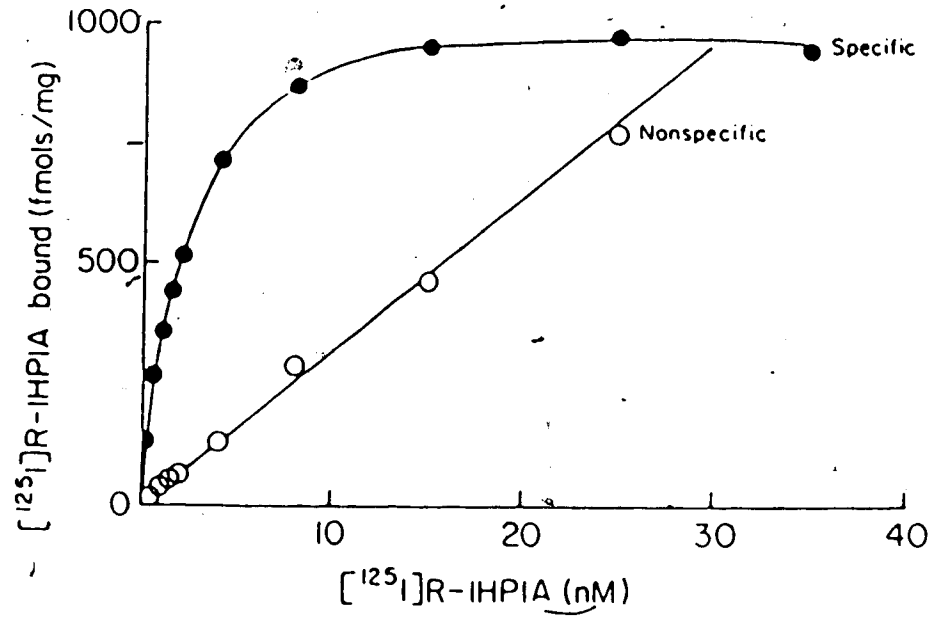
Fig. 10 shows that the nonspecific binding was linear up to a radioligand concentration in the incubation of at least 35 nM. The specific binding component was saturable with a K_D of 2.1 nM and B_{max} of 1080 fmols/mg as estimated by the nonlinear curve-fitting approach. Scatchard analysis of the specific binding data revealed a linear plot (Fig. 10, inset 1) suggesting the presence of a single population of binding sites with a K_D = 1.85 nM and B_{max} = 1060 fmols/mg, both values being very similar to those obtained by the nonlinear curve fitting approach. For Scatchard analysis, the free concentration of the radioligand was corrected for depletion by ligand bound to the membrane. The slope of the Hill plot (Fig. 10, inset 2) was 0.9, suggesting the absence of co-operativity.

3.3.7.3 Displacement analysis

Various agonists with low to high nanomolar affinities for adenosine receptors displaced the specific binding of [^{125}I]R-IHPIA (Fig. 11). The rank order of potency in this set of experiments was R-PIA>R-HPIA>NECA>R-IHPIA>2-chloroadenosine>S-PIA>S-HPIA>MECA, which is typical of binding at adenylate cyclase-coupled A_1 receptors. In the same set of experiments, the R-enantiomers of PIA and HPIA were about 10-fold more potent than the corresponding S-enantiomers. Theophylline (10 mM) displaced the binding to the same extent as 10 μM R-PIA. Adenosine receptor antagonists such as theophylline and SPT displaced the binding with lower affinities than the agonists, as would be expected for binding at adenosine receptors (Fig. 12). Table

Fig. 10. Concentration dependence of [^{125}I]R-IHPIA binding to rat brain membranes. Binding of [^{125}I]R-IHPLA to rat brain membranes was measured at 30°C for 2 h. Nonspecific binding was determined in the presence of 10 μM R-PIA. Values are a mean of triplicate determinations with standard deviations at most points between 2 – 8%. Insets: The specific binding data was linearized on Scatchard (1) and Hill (2) plots. B is the specific binding at various concentrations of the radioligand. B_{max} (1060 fmols/mg) was estimated from plot 1 (r=0.98), the slope of which also provided the affinity of the radioligand (K_D=1.85 nM). The slope of plot 2 provided the estimate for the Hill coefficient (n^H=0.9).


1
Bmax = 1060 fmols/mg
K_D = 1.85 nM
B/F
B
2
Log F
Log[B/(Bmax-B)]
n^H = 0.9


Specific
Nonspecific
[125I]R-IHPIA bound (fmols/mg)
[125I]R-IHPIA (nM)

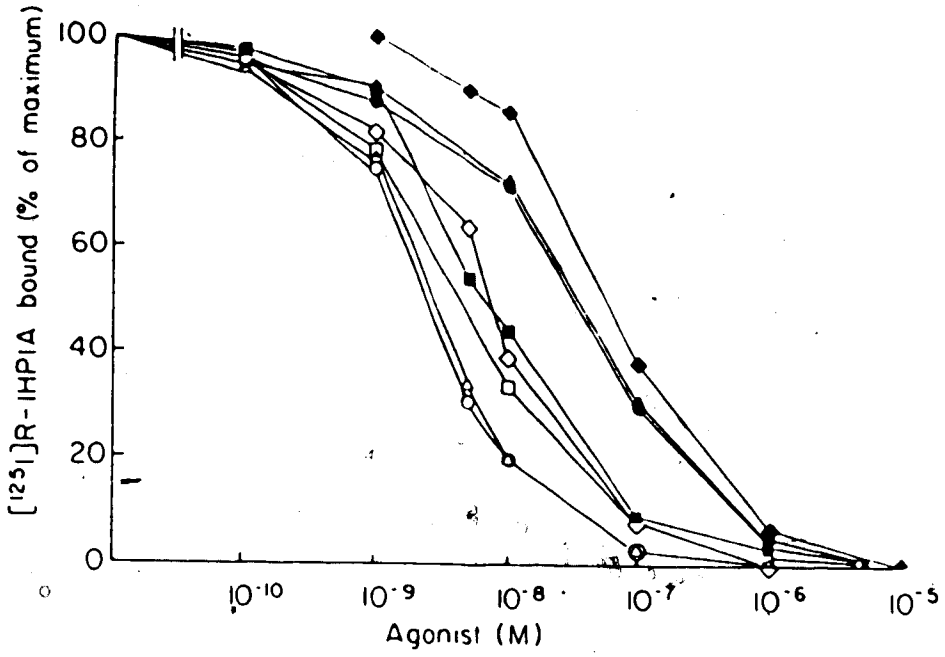


Fig. 11. Displacement of specifically bound [^{125}I]K-IHPIA by various agonists of adenosine receptors in rat brain membranes. Rat brain membranes were incubated with [^{125}I]K-IHPIA (170 pM) at 30°C for 2 h in the presence of various concentrations of different agonists. K-PIA (○), K-HPIA (△), NECA (□), 2-chloroadenosine (■), K-IHPIA (◇), S-PIA (●), S-HPIA (▲), MECA (◆). Values are a mean of triplicate determinations with standard deviations at most points between 5 - 14%.

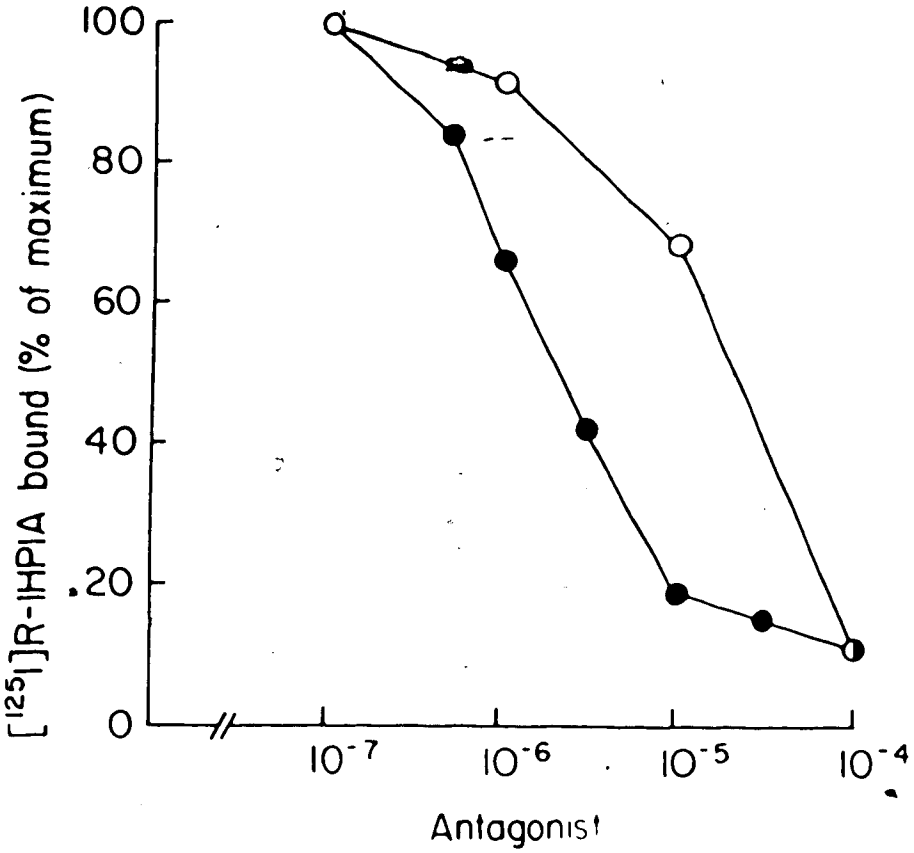


Fig. 12. Displacement of specifically bound [^{125}I]R-IHPIA (170 pM) by antagonists at adenosine receptors in rat brain membranes. The protocol for the binding was as described in Fig. 11. SPT (●), theophylline (○). Values are a mean of triplicate determinations with maximum standard deviations of 12%.

Table 4. Comparison of the inhibitory constant (Ki)* of various inhibitors of [^{125}I]R-IHPIA binding in rat brain membranes

Inhibitor	Ki (nM)	n_H
R-PIA	1.58	0.92
R-HPIA	1.65	0.98
[illegible]ECA	3.9	0.77
R-IHPIA	4.9	0.78
2-chloroadenosine	5.8	0.86
S-PIA	18.5	0.78
S-HPIA	21.6	0.79
MECA	51.4	0.92
SPT	5,200	0.98
Theophylline	14,000	0.99

*The Ki values were estimated using the equations provided by Linden (1982; see section 2.8.1.1.3). Each value is from a single assay performed in triplicate. The value of the regression coefficient r (Hill plots) ranged between 0.93 and 0.998.

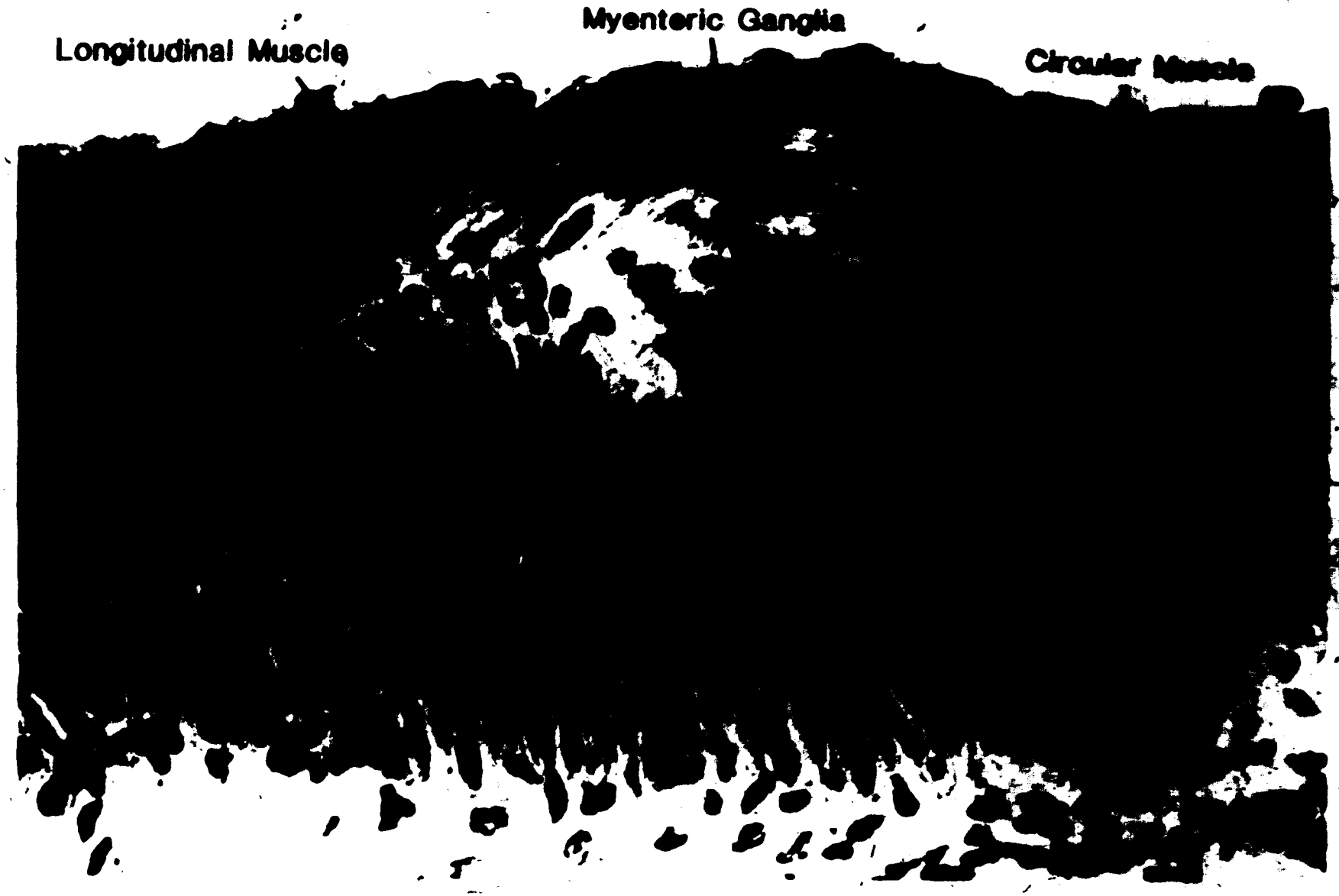
4 summarizes the values for the inhibitory constant (Ki) and the Hill slopes (n^H). Hill plots of the displacement data for various compounds tested had slopes near one (usually about 0.8).

3.4 Radioligand binding using small-intestinal membranes from rabbit

3.4.1 General comments

When visualized under a light microscope, a thin section of the longitudinal muscle revealed that the muscle strip was contaminated with a certain mass of circular muscle and myenteric ganglia (Fig. 13). Thus the membrane preparation contained plasma membranes from the longitudinal muscle cells, the circular muscle cells and the neuronal cells of the myenteric ganglia. In a preliminary experiment it was observed that the basal, GTP- and forskolin-stimulated adenylate cyclase activity (plasma-membrane marker) was highest in the P_3 pellet; the P_1 pellet showed the least enzyme activity (Fig. 14). Therefore, the P_3 pellet was used in all binding assays except for the comparison of the filtration and centrifugation assays, where the particulate fraction corresponding to the P_2 pellet was used. This was deemed necessary since the bound and the free radioligand were separated in a centrifugation assay at a g-force corresponding to that for the P_2 pellet. Preliminary experiments using DTT (1 mM) in the buffer during membrane preparation revealed that DTT did not modify the specific binding of either [^{125}I]R-IHPIA or [^{3}H]NECA. Also, when DTT (1 mM, final) was included in the binding incubations, the specific binding of both radioligands was slightly lower. DTT was

Fig. 13. Micrograph of the longitudinal muscle of small intestine of rabbit. Transverse section.


Longitudinal Muscle
Myenteric Ganglia
Circular Muscle

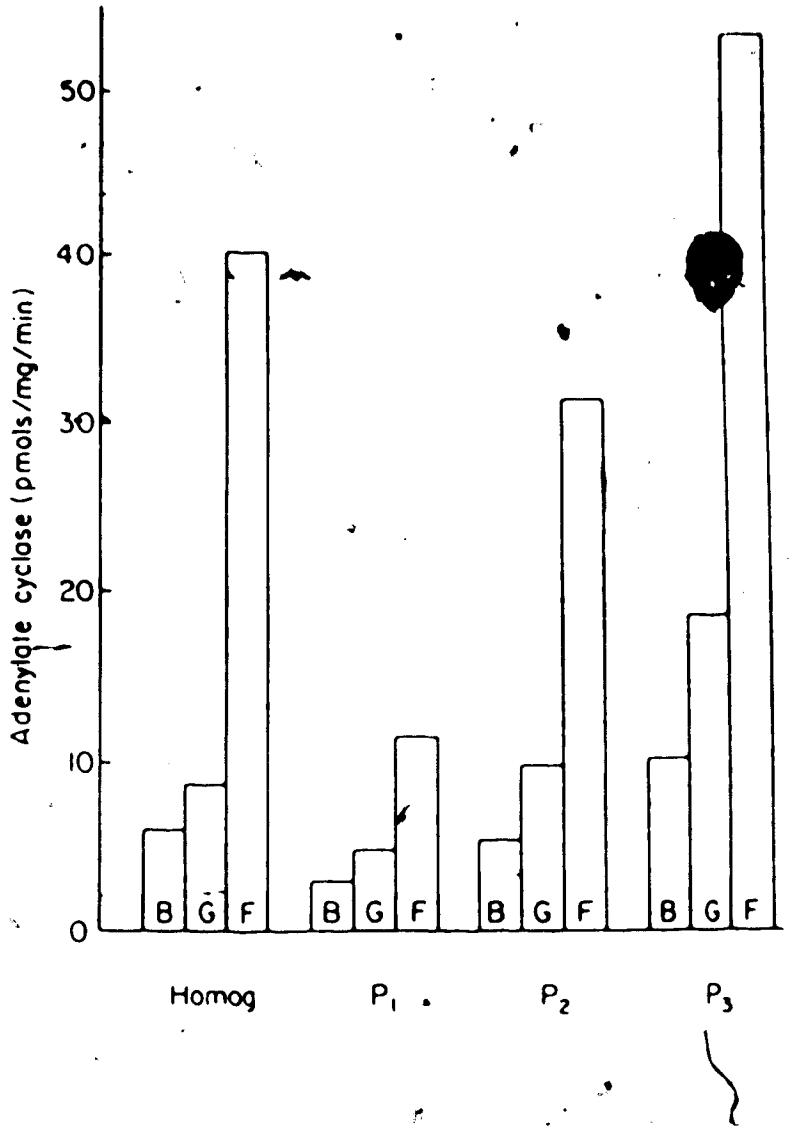


Fig. 14. Subcellular distribution of adenylate cyclase activity in particulate fractions from rabbit small-intestinal longitudinal muscle. Basal (B), and GTP (G)- and forskolin (F)-stimulated adenylate cyclase activity was measured in four particulate fractions of the longitudinal muscle at 37°C for 20 min. Values are a mean of triplicate determinations with standard deviations at most points between 8 - 14%.

consequently, omitted in subsequent binding assays.

3.4.2 Measurement of [^{125}I]R-IHPIA and [^{3}H]R-PIA binding

3.4.2.1 Identification of the bound radioactivity ([^{125}I]R-IHPIA)

Rabbit intestinal membranes (500 µg) were incubated with 500 pM [^{125}I]R-IHPIA under standard assay conditions and the bound radioactivity extracted as described under section 3.3.2. The ethylacetate extract of the filter paper-bound radioactivity revealed a single spot on the autoradiogram which co-chromatographed with the radioligand from the stock ligand preparation.

3.4.2.2 Comparison of the filtration and centrifugation assays

Table 5 compares the total and nonspecific binding to the pellet P_2 at 20°C for both radioligands. In both cases, the specific binding as observed in a single assay was higher when a filtration assay was used. Therefore, in all subsequent assays the bound and free radioligand were separated by the filtration method.

3.4.2.3 Effect of washing

At the end of the incubation time, the incubations were filtered and the filters were washed 1 to 5 times with 5 ml of ice-cold buffer each time. When [^{125}I]R-IHPIA was used as the radioligand, the specific binding was similar after 1 or 2 washes but decreased considerably when the number of washes was higher than this. When [^{3}H]R-PIA was used as the radioligand, the specific binding was highest after a

Table 5. Comparison of [^{125}I]R-IHPIA and [^{3}H]R-PIA binding to rabbit intestinal membranes in filtration and centrifugation assays

Radioligand	fmols bound per incubation protein					
	Filtration assay			Centrifugation assay		
	Total	Nonspecific	Specific	Total	Nonspecific	Specific
[^{125}I]R-IHPIA	2.3 ± 0.12	1.6 ± 0.26	0.7	3.9 ± 0.064	3.3 ± 0.16	0.6
[^{3}H]R-PIA	43.6 ± 1.38	17.7 ± 3.1	25.9	52.0 ± 0.69	39.4 ± 4.8	12.6

Concentration of [^{125}I]R-IHPIA = 230 pM
Concentration of [^{3}H]R-PIA = 30 nM
The values represent a mean of triplicate determinations with Standard Deviations.

single wash and decreased as the number of washes was increased to 5. The filter papers were therefore washed once only with 5 ml of the ice-cold buffer in all subsequent experiments using both radioligands.

3.4.2.4 Effect of pretreatment with adenosine deaminase

Fig. 15 shows that pretreatment of the membranes with 1 to 4 International Units of adenosine deaminase per ml of membrane suspension (to deaminateendogenous adenosine) did not affect the level of specific binding of [^{125}I]R-IHPIA. The membranes were therefore used in binding assays without pretreatment with adenosine deaminase.

3.4.2.5 Effect of varying the protein concentration

Both the total and nonspecific binding was linear up to 260 μg ([^{125}I]R-IHPIA) and 316 μg ([^{3}H]R-PIA) of protein per incubation (Fig. 16A,B). Eighty to 120 μg of membrane protein per incubation was used in [^{125}I]R-IHPIA binding assays and 100 - 140 μg in [^{3}H]R-PIA binding assays.

3.4.2.6 Effect of temperature

In a preliminary assay it was observed that the specific binding of [^{125}I]R-IHPIA binding is higher at 20°C than at 10°C and 37°C (Fig. 17A). In a similar assay the specific binding of [^{3}H]R-PIA was maximum between 2 and 20°C and declined at higher temperatures (Fig. 17B). A temperature of 20°C was chosen for all subsequent assays using both radioligands, unless stated otherwise in the figure legends.

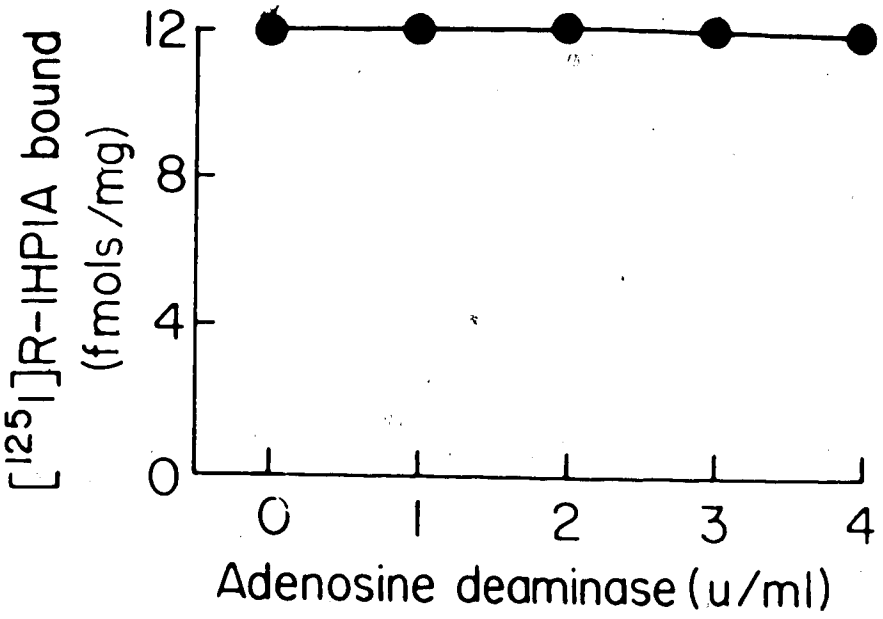
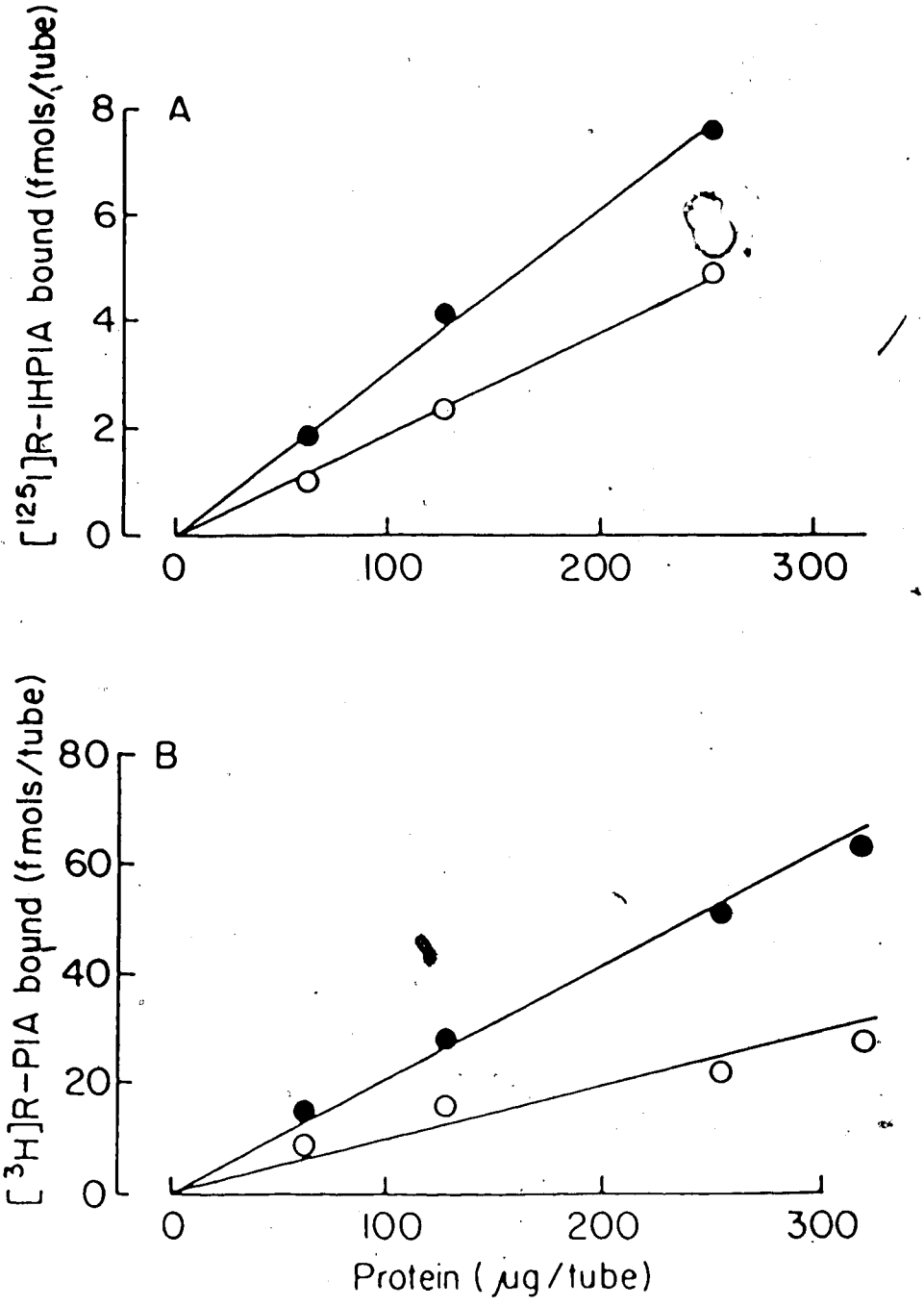


Fig. 15. Effect of pretreatment with adenosine deaminase on [^{125}I]R-IHPIA binding to longitudinal-muscle membranes of rabbit small intestine. Membranes, preincubated with various concentrations of adenosine deaminase at 37°C for 30 min, were used in a standard assay with 190 pM radioligand at 20°C for 10 min. Values are means of triplicate determinations. Standard deviations were below 12%.

Fig. 16. Effect of variation in the protein concentration on (A) [^{125}I]R-IHPIA and (B) [^{3}H]R-PIA binding to longitudinal-muscle membranes of rabbit small intestine. A. Membranes (62 - 256 μg per incubation) were used in a standard binding assay with 400 pM [^{125}I]R-IHPIA at 20°C for 10 min, in the presence (○) and absence (●) of 100 μM R-PIA. B. Same as in A but using 20 nM [^{3}H]R-PIA and 62 - 326 μg protein per incubation at 20°C for 10 min, in the presence (○) andabsence (●) of 500 μM R-PIA. All values are means of duplicate determinations. Standard deviation at most points was between 6 - 14%.


[125I]R-IHPIA bound (fmols/tube)
A
8
6
4
2
0
0
100
200
300
[3H]R-PIA bound (fmols/tube)
B
80
60
40
20
0
0
100
200
300
Protein (µg / tube)

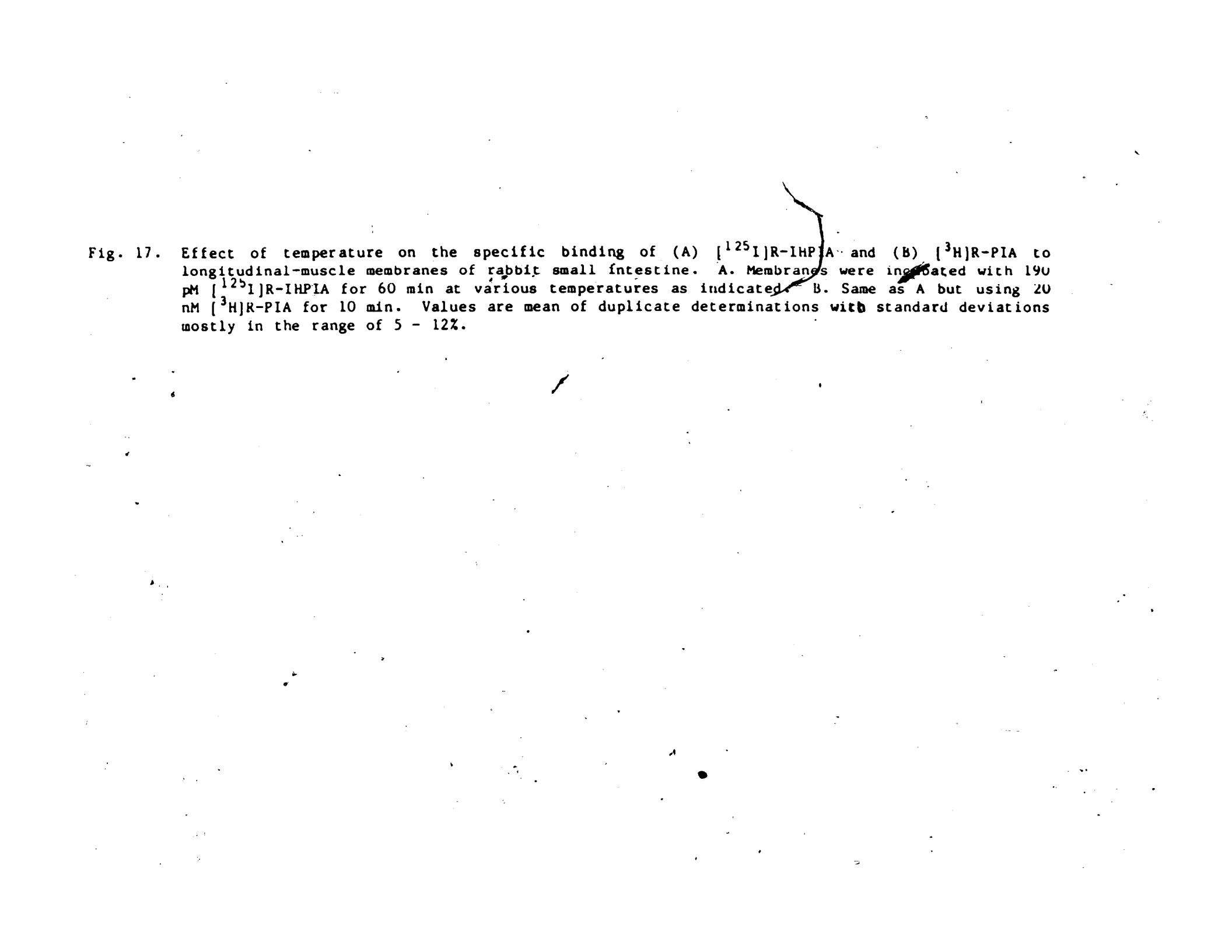
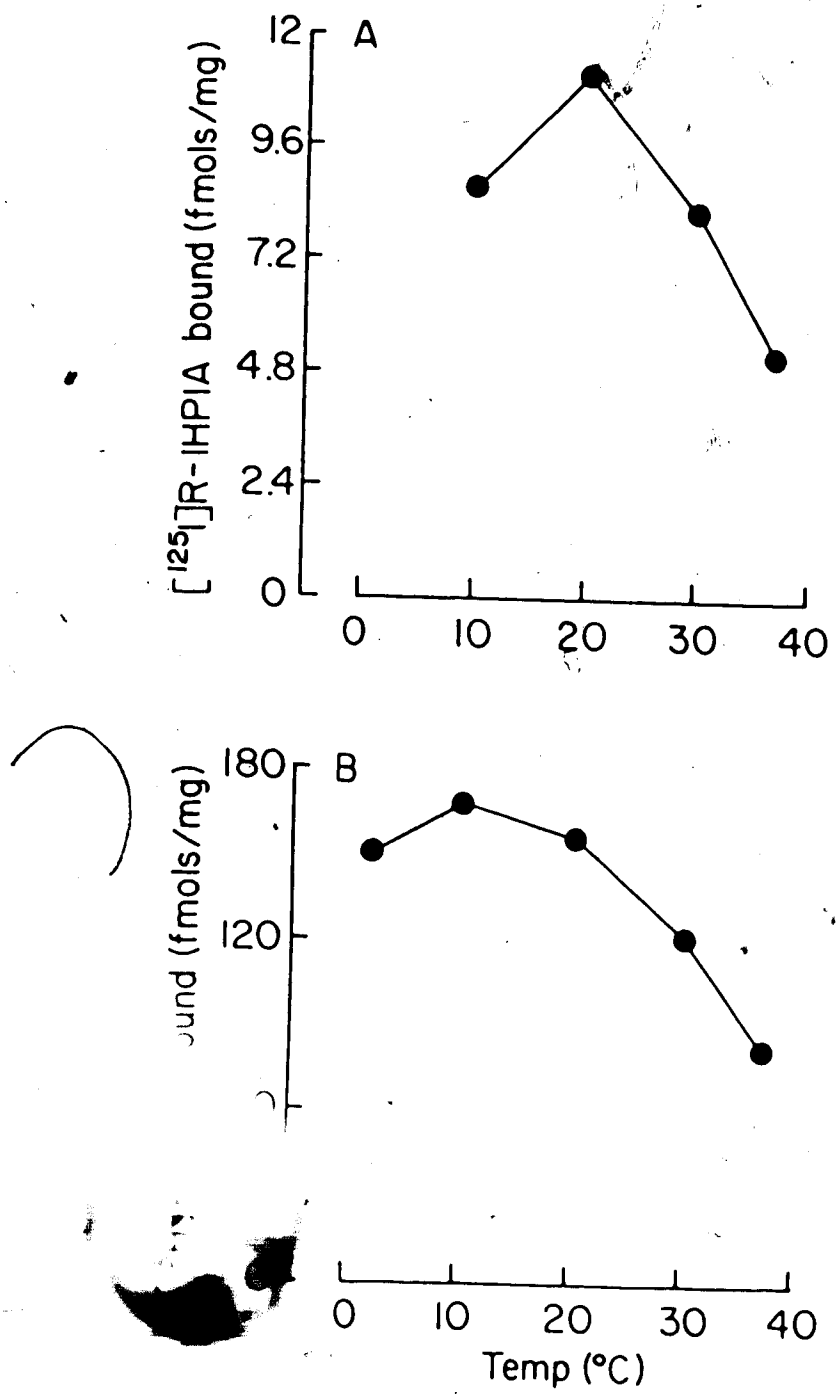


Fig. 17. Effect of temperature on the specific binding of (A) [^{125}I]R-IHPIA and (B) [^{3}H]R-PIA to longitudinal-muscle membranes of rabbit small intestine. A. Membranes were incubated with 190 pM [^{125}I]R-IHPIA for 60 min at various temperatures as indicated. B. Same as A but using 20 nM [^{3}H]R-PIA for 10 min. Values are mean of duplicate determinations with standard deviations mostly in the range of 5 - 12%.


A
12
9.6
7.2
4.8
2.4
0
[125I]R-IHPIA bound (fmols/mg)
0
10
20
30
40
B
180
120
und (fmols/mg)
0
10
20
30
40
Temp (°C)

3.4.2.7 Effect of pH

No appreciable change in [^{3}H]R-PIA binding (both specific and nonspecific) was evident over the pH range (7 - 9) of Tris-buffer (Fig. 18). The binding assays were performed using Tris buffer of pH 7.4.

3.4.2.8 Effect of ion concentrations

Final incubation concentrations of 1 - 10 mM Ca^{2+}, Mg^{2+} and Na^{+} did not affect the specific binding of [^{125}I]R-IHPIA as the radioligand (Fig. 19). However, in the same experiment, Mg^{2+} and Na^{+} at higher concentrations (50 mM) each decreased the total binding by 37%.

3.4.2.9 Kinetics of binding of the radioligands

Specific binding of [^{125}I]R-IHPIA equilibrated within less than 5 min and remained stable up to at least 15 min at 20°C. Upon addition of unlabelled R-PIA (100 μM), the site bound [^{125}I]R-IHPIA was rapidly displaced by nonisotopic R-PIA with half maximal dissociation of binding in about 3 min (Fig. 20A). Both the association of [^{3}H]R-PIA and its dissociation from the specific binding sites at 2°C was very rapid. The specific binding of [^{3}H]R-PIA equilibrated within less than 3 min and remained stable for up to at least 25 min. The dissociation of the radioligand was very rapid with half maximal dissociation time of less than 20 sec (Fig. 20B). Approximately 18% of the specifically bound radioligand did not dissociate in the presence of 100 μM R-PIA. Since nonspecific binding was estimated

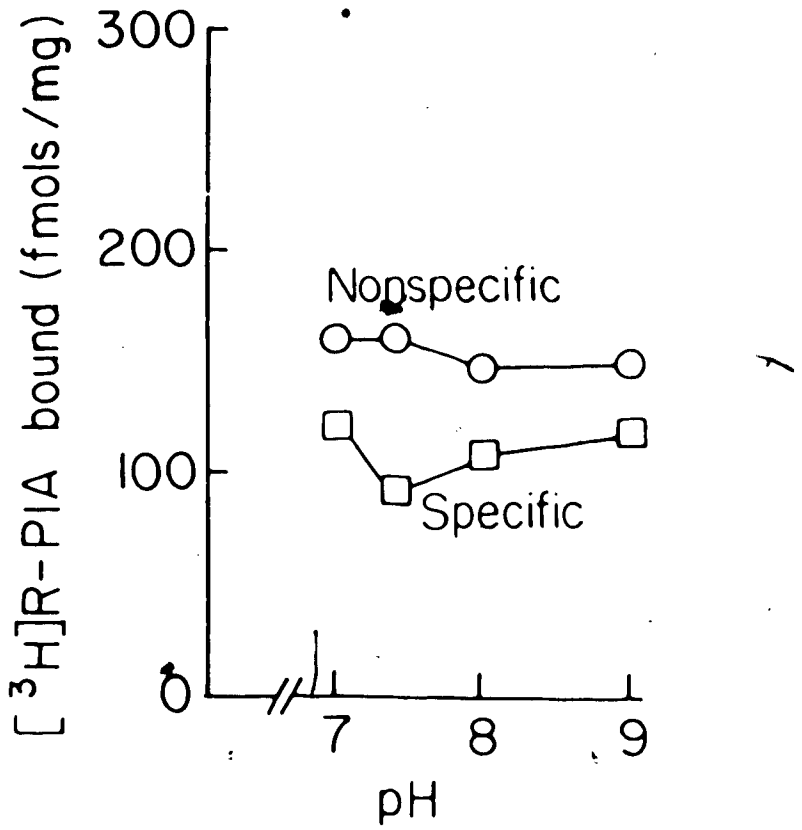


Fig. 18. Effect of pH variation on [^{3}H]R-PIA binding to longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with 20 nM radioligand at 20°C for 10 min. The nonspecific binding was estimated in the presence of 500 μM R-PIA. Values are mean of triplicate determinations with standard deviations of less than 15%.

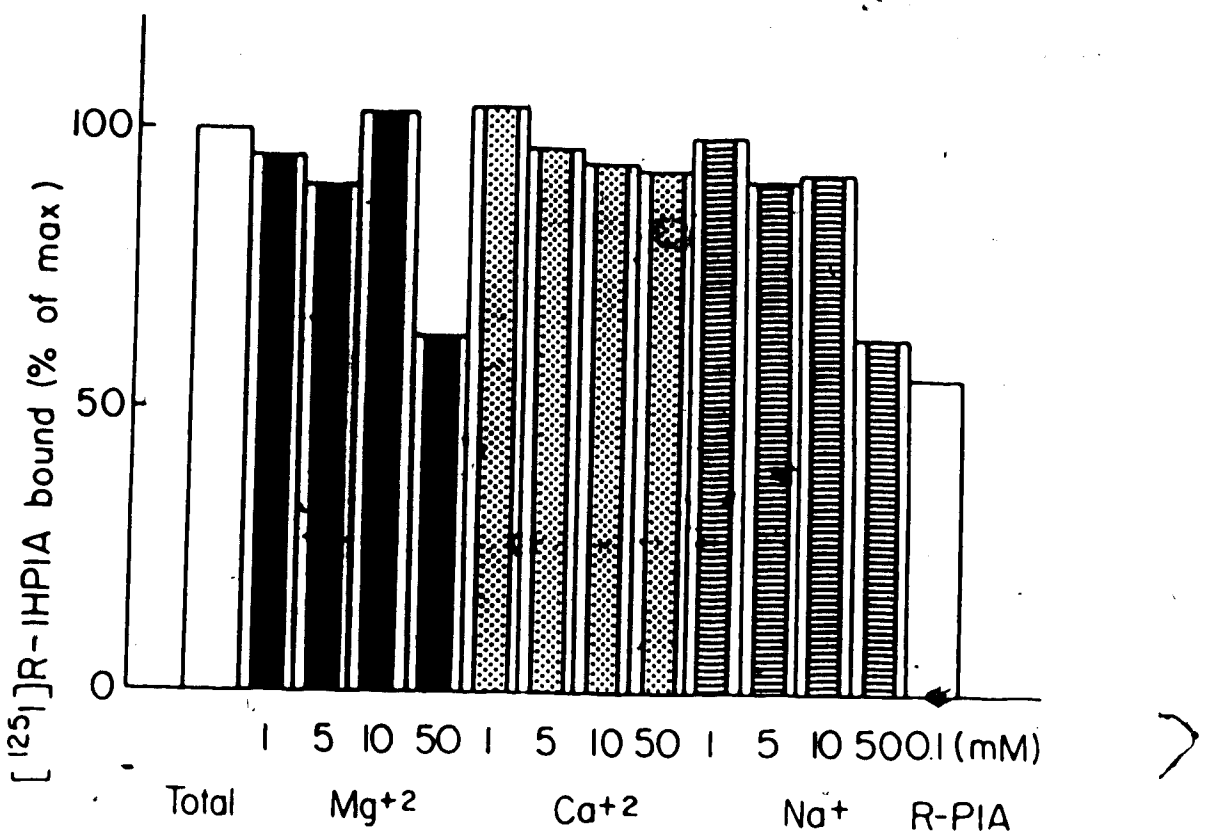
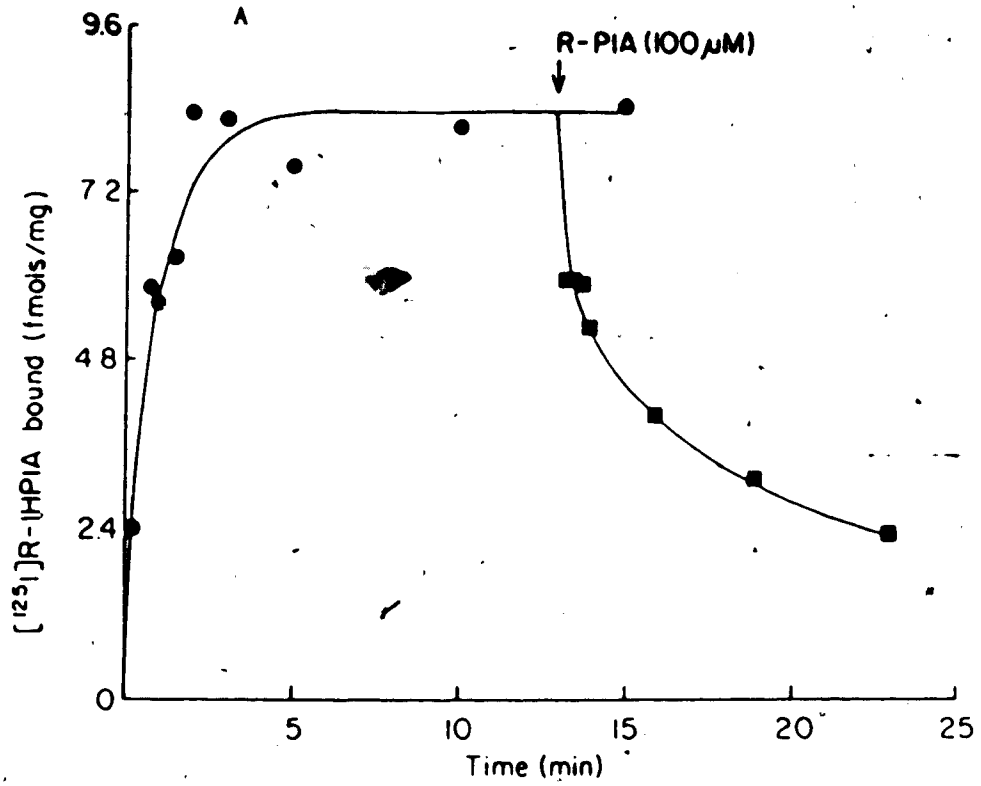
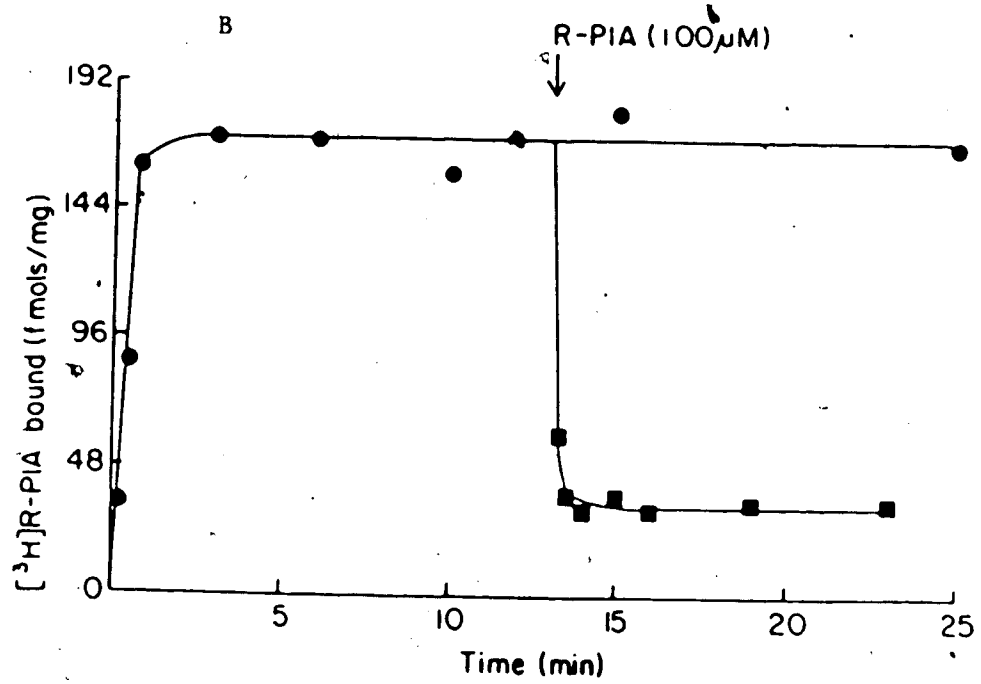


Fig. 19. Effect of ion concentrations on [^{125}I]R-IHPIA binding to longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with 330 pM [^{125}I]R-IHPIA at 20°C for 10 min. Values are mean of triplicate determinations with standard deviations of less than 16%.

Fig. 20. Time course of association and dissociation of (A) [^{125}I]R-IHPIA and (B) [^{3}H]R-PIA specific binding to longitudinal-muscle membranes of rabbit small intestine. A. Membranes were incubated with 190 pM radioligand at 20°C. 450-µl aliquots were withdrawn at various times up to 15 min (abscissa) and filtered as described under section 2.5.2.6. Dissociation was induced by adding R-PIA to the incubation at a final concentration of 100 µM. B. Membranes were incubated as above with 20 nM [^{3}H]R-PIA at 0°C. Dissociation was induced with R-PIA at a final concentration of 500 µM. Values are mean of 2 assays (A) and a single assay (B) each performed in duplicate with standard deviations of 9 - 15% at most points.


A
R-PIA (100μM)
9.6
7.2
4.8
2.4
0
[125I]R-HPIA bound (fmols/mg)
5
10
15
20
25
Time (min)

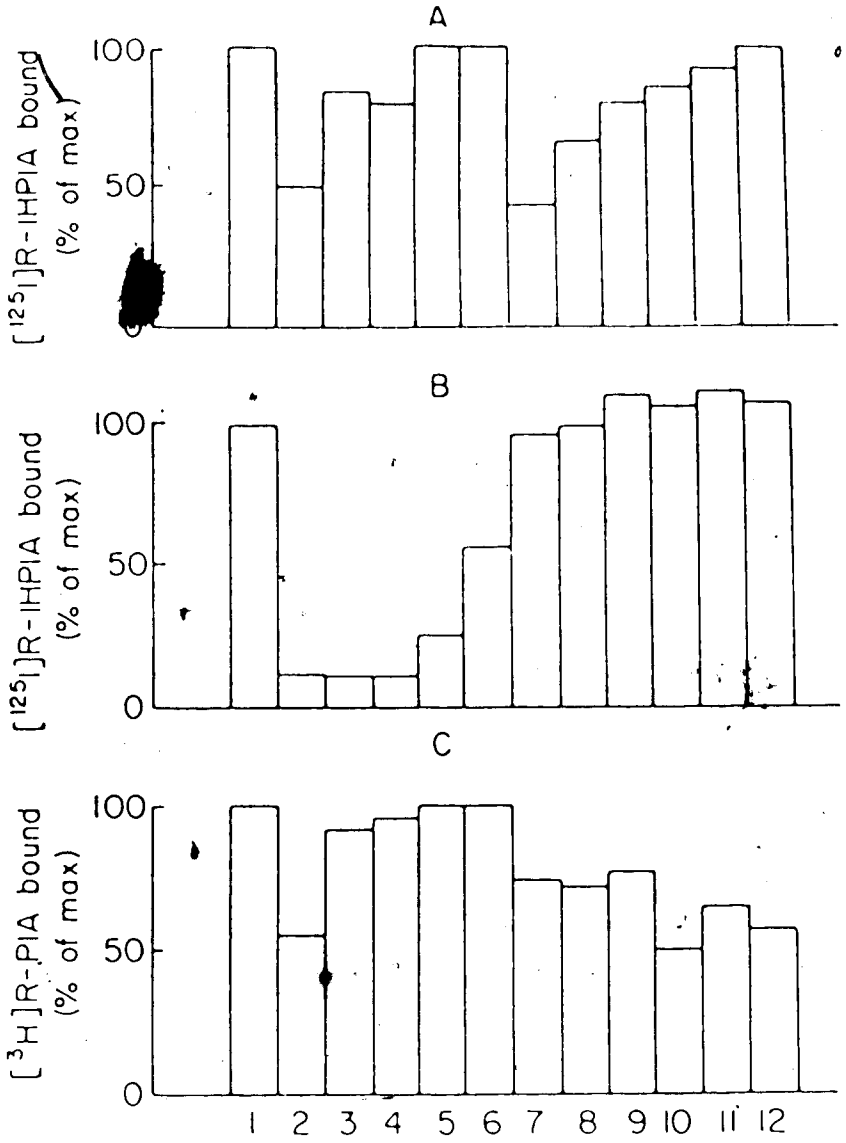

B
R-PIA (100μM)
192
144
96
48
0
[3H]R-PIA bound (fmols/mg)
5
10
15
20
25
Time (min)

using 500 μM R-PIA, it is possible that the use of 5-fold lower concentration of R-PIA in the dissociation assay, did not allow complete dissociation of the specifically bound radioligand. No kinetic constants were derived from these data using either radioligand.

3.4.2.10 Displacement analysis

Fig. 21 compares the effect of various known agonists and antagonists of adenosine receptors on the total binding of [^{125}I]R-IHPIA and [^{3}H]R-PIA to rabbit small-intestinal membranes with that of [^{125}I]R-IHPIA binding to rat brain membranes. Among the various compounds used, each at 100 μM in a single assay performed in triplicate, only R-PIA appreciably displaced the total binding to small-intestinal membranes by 50% for [^{125}I]R-IHPIA and 45% for [^{3}H]R-PIA. Other compounds such as NECA, 2-chloroadenosine, and SPT were either only weakly effective or totally inactive on [^{125}I]R-IHPIA and [^{3}H]R-PIA binding sites. On the other hand, these compounds displaced [^{125}I]R-IHPIA binding to rat brain membranes very effectively (by 76 - 88%). The lack of specificity of the binding of [^{125}I]R-IHPIA and [^{3}H]R-PIA to the small-intestinal membranes is further demonstrated by the use of compounds, each at 100 μM, which are known antagonists at other receptor systems. Compounds such as haloperidol (dopamine receptor antagonist), methysergide (serotonin receptor antagonist) and cimetidine (histamine-H_2 receptor antagonist) reduced the binding of [^{125}]R-IHPIA (by 20 - 57%) and [^{3}H]R-PIA

Fig. 21. Comparison of the displacement by various inhibitors of [^{125}I]R-IHPIA and [^{3}H]R-PIA binding to longitudinal-muscle membranes of rabbit small intestine with that of [^{125}I]R-IHPIA binding to rat brain membranes. [^{125}I]R-IHPIA was incubated with longitudinal-muscle membranes at 20°C for 10 min (Panel A) and with rat brain membranes at 30°C for 2 h (Panel B). [^{3}H]R-PIA was incubated with longitudinal-muscle membranes at 20°C for 10 min (Panel C). The binding in all three cases was measured either (1) in the absence of any inhibitor or (2) in the presence of R-PIA, (3) 2-chloroadenosine, (4) NECA, (5) SPT (6), Gpp(NH)p, (7) haloperidol, (8) methysergide, (9) cimetidine, (10) Mepyramine, (11) atropine and (12) hexamethonium (12). All inhibitors were used at the same final concentration of 100 µM. Values are mean of triplicate determinations with 4 - 12% standard deviations. [^{125}I]R-IHPIA: 252 pM (longitudinal-muscle membranes, A) and 170 pM (rat brain membranes, B). [^{3}H]R-PIA: 30 nM (C).


A
100
50
0
[125I]R-IHPIA bound
(% of max)
B
100
50
0
[125I]R-IHPIA bound
(% of max)
C
100
50
0
[3H]R-PIA bound
(% of max)
1 2 3 4 5 6 7 8 9 10 11 12

(by 23 - 27%) to the small-intestinal membranes. Furthermore, compounds such as mepyramine (histamine-H_1 receptor antagonist), atropine (muscarinic receptor antagonist) and hexamethonium (ganglionic nicotinic receptor blocker) reduced the binding of [^{3}H]R-PIA by 35 - 50%. By contrast, none of these compounds (100 μM each) was effective against [^{125}I]R-IHPIA binding to rat brain membranes. The binding sites for both [^{125}I]R-IHPIA and [^{3}H]R-PIA in the small intestinal membranes therefore appear to be nonreceptor sites not involved in adenosine-induced relaxation of the small intestine.

3.4.3 Measurement of [^{3}H]NECA binding

3.4.3.1 Comparison of the filtration and centrifugation assays

The specific binding was about 2.7 fold higher when the bound and free radioactivity was separated by filtration as opposed to centrifugation (Table 6). Filtration binding was therefore used in all subsequent assays.

3.4.3.2 Effect of washing

The filter papers were washed 1 to 5 times with 5 ml of ice-cold buffer each time. The specific binding was maximal after a single wash and declined with increase in the number of washes. The number of washes had only a marginal effect on the nonspecific binding. The filter papers were washed once with 5 ml of ice cold buffer in all subsequent experiments (data not shown).

Table 6. Comparison of [^{3}H]NECA binding to rabbit intestinal membranes in filtration and centrifugation assays

fmols bound per incubation protein					
Filtration assay			Centrifugation assay		
Total	Nonspecific	Specific	Total	Nonspecific	Specific
154.7 ± 5.9	25.0 ± 2.6	129.7	197 ± 10.1	148.7 ± 13.9	48.3

Concentration of [^{3}H]NECA = 30 nM

The values represent a mean of three experiments each performed in triplicate.

3.4.3.3 Effect of pretreatment with adenosine deaminase

Pretreatment of the membrane preparation with up to 5 U of adenosine deaminase per ml of the membrane suspension for 30 min at 37°C did not increase the specific binding (Fig. 22). Adenosine deaminase was therefore omitted in subsequent assays.

3.4.3.4 Effect of varying the protein concentration

Both the total and nonspecific binding was linear up to 316 µg protein per incubation (Fig. 23). One hundred to 150 µg protein per incubation was used in all subsequent assays.

3.4.3.5 Effect of temperature

The specific binding of [^{3}H]NECA declined progressively as the temperature was increased from 2 to 37°C, with half maximal specific binding at about 10°C (Fig. 24). In all subsequent assays a temperature of 2°C was used.

3.4.3.6 Effect of pH

There was no effect on either the nonspecific binding (measured in the presence of 500 µM NECA) or the specific binding of [^{3}H]NECA to the longitudinal-muscle membrane when the pH of the incubation buffer varied between 7 and 9 (Fig. 25).

3.4.3.7 Kinetics of the binding

The specific binding of [^{3}H]NECA equilibrated within 15 min and remained stable for up to at least 30 min (Fig. 26). The forward rate

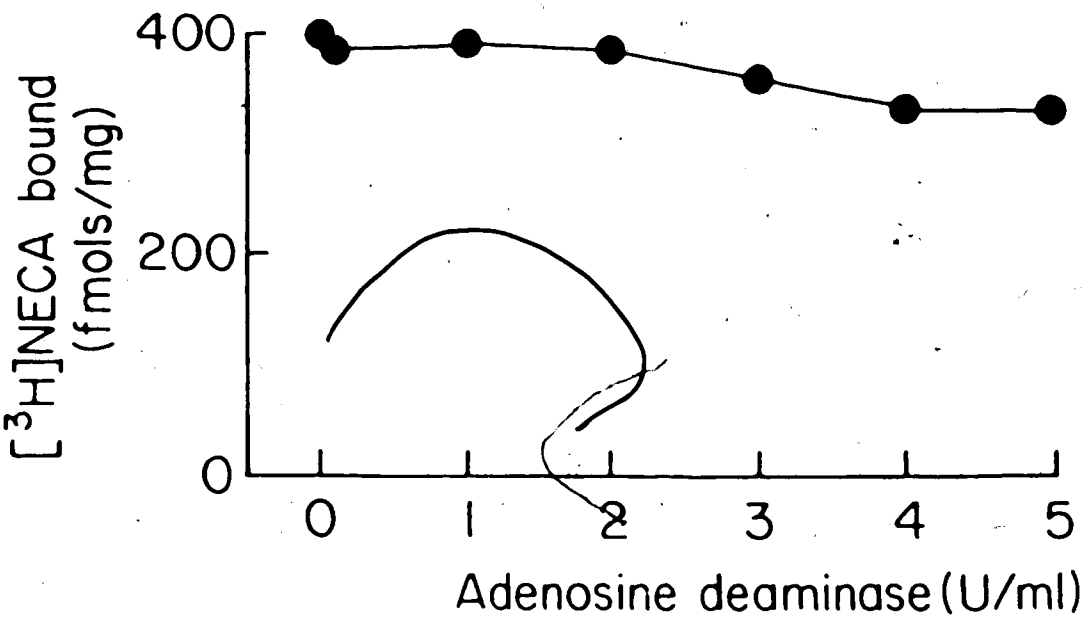


Fig. 22. Effect of pretreatment with adenosine deaminase on [^{3}H]NECA binding to longitudinal muscle membranes of rabbit small intestine. The membranes, preincubated with various concentrations of adenosine deaminase at 37°C for 30 min, were used in a standard binding assay with 20 nM radioligand at 2°C for 20 min. Values are means of triplicate determinations with standard deviations of less than 16%.

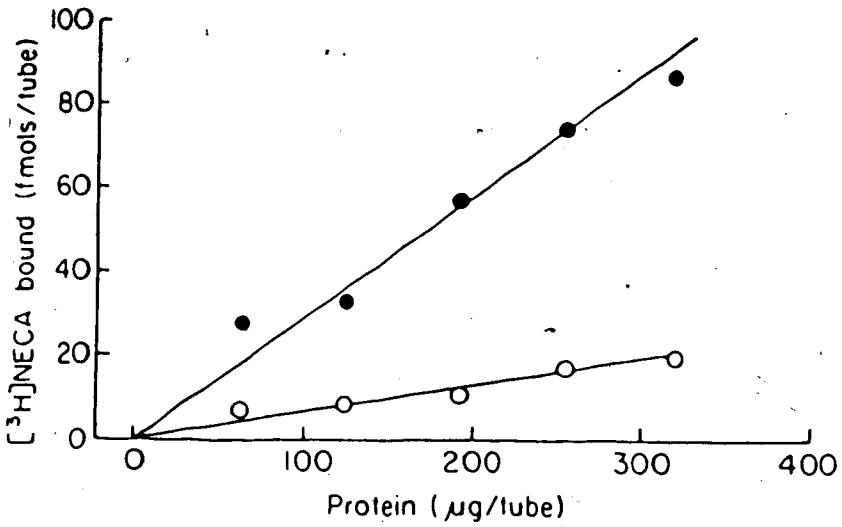


Fig. 23. Effect of variation in the protein concentration on [^{3}H]NECA binding to longitudinal-muscle membranes of rabbit small intestine. Membranes (62 - 326 µg protein per incubation) were used in a standard binding assay with ~10 nM radioligand at 2°C for 20 min in the presence (○) and absence (●) of 500 µM NECA. Values are means of duplicate determinations. Standard deviations were in the range of 8 - 14%.

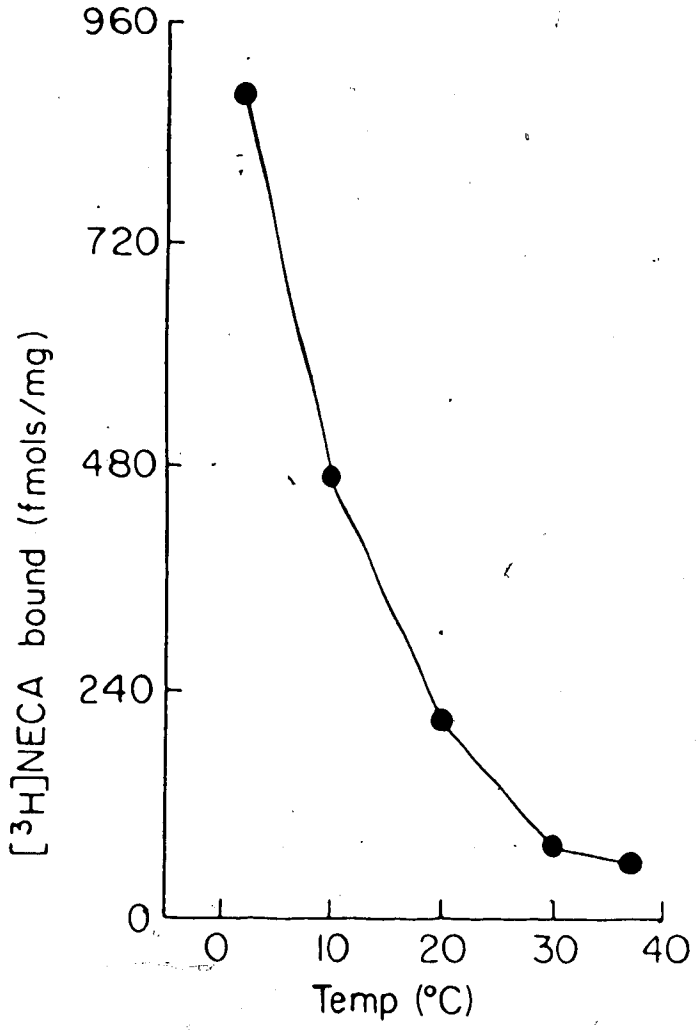


Fig. 24. Effect of temperature on the specific binding of [^{3}H]NECA to longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with 30 nM radioligand for 20 min at various temperatures as indicated. Values are means of triplicate determinations with standard deviations of less than 11%.

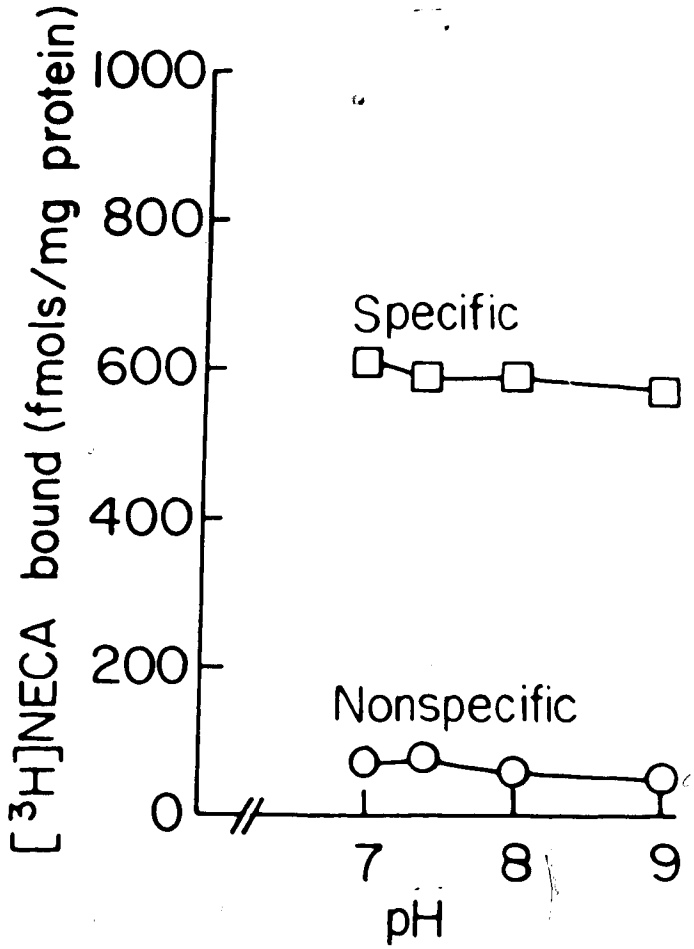


Fig. 25. Effect of pH variation on the specific binding of [^{3}H]NECA longitudinal-muscle membranes of rabbit small intestine. The membranes were incubated with 20 nM radioligand at 2°C for 20 min. The nonspecific binding was estimated in the presence (O) of 500 μM NECA. Values are means of triplicate determinations. Standard deviations were less than 14%.

constant as estimated by the pseudo first order was equation 6.3×10^5 M^{-1} min^{-1}. Upon addition of unlabelled NECA (100 μM), the specific binding dissociated very rapidly with the loss of half maximal binding in less than 20 sec and almost all the specifically bound radioligand dissociated within 10 min of the addition of cold NECA. No kinetic constants were derived from these data.

3.4.3.8 Determination of the binding constants

3.4.3.8.1 Saturation analysis

The nonspecific binding was considerably lower than the total binding (24% of total binding at the K_D, Fig. 27). The binding constants as derived from the nonlinear curve fitting approach are: B_{max} = 16.9 pmols/mg protein retained on the filters and K_D = 0.55 μM. The graphical Scatchard analysis of the specific binding data was consistent with binding to a single class of sites and provided essentially similar parameters (B_{max} = 15.7 pmols/mg and K_D = 0.45 μM, Fig. 27, inset 1). The Hill plot of the specific binding data provided an estimation of the Hill coefficient, n^H = 1.05 (Fig. 27, inset 2) suggesting a lack of co-operativity.

3.4.3.8.2 Displacement analysis

Various 5'-modified derivatives of adenosine that decrease the spontaneous contractility of rabbit gut (Table 8) were among the most effective (low micromolar Ki) inhibitors of [^{3}H]NECA binding (Fig. 28). 2-Choroadenosine was about 2-fold less potent than NECA.

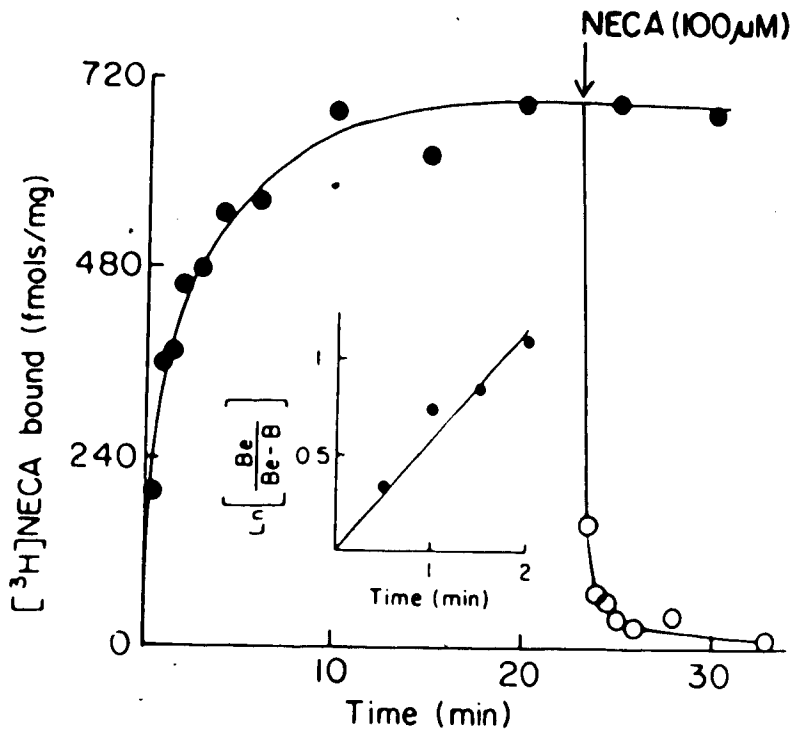
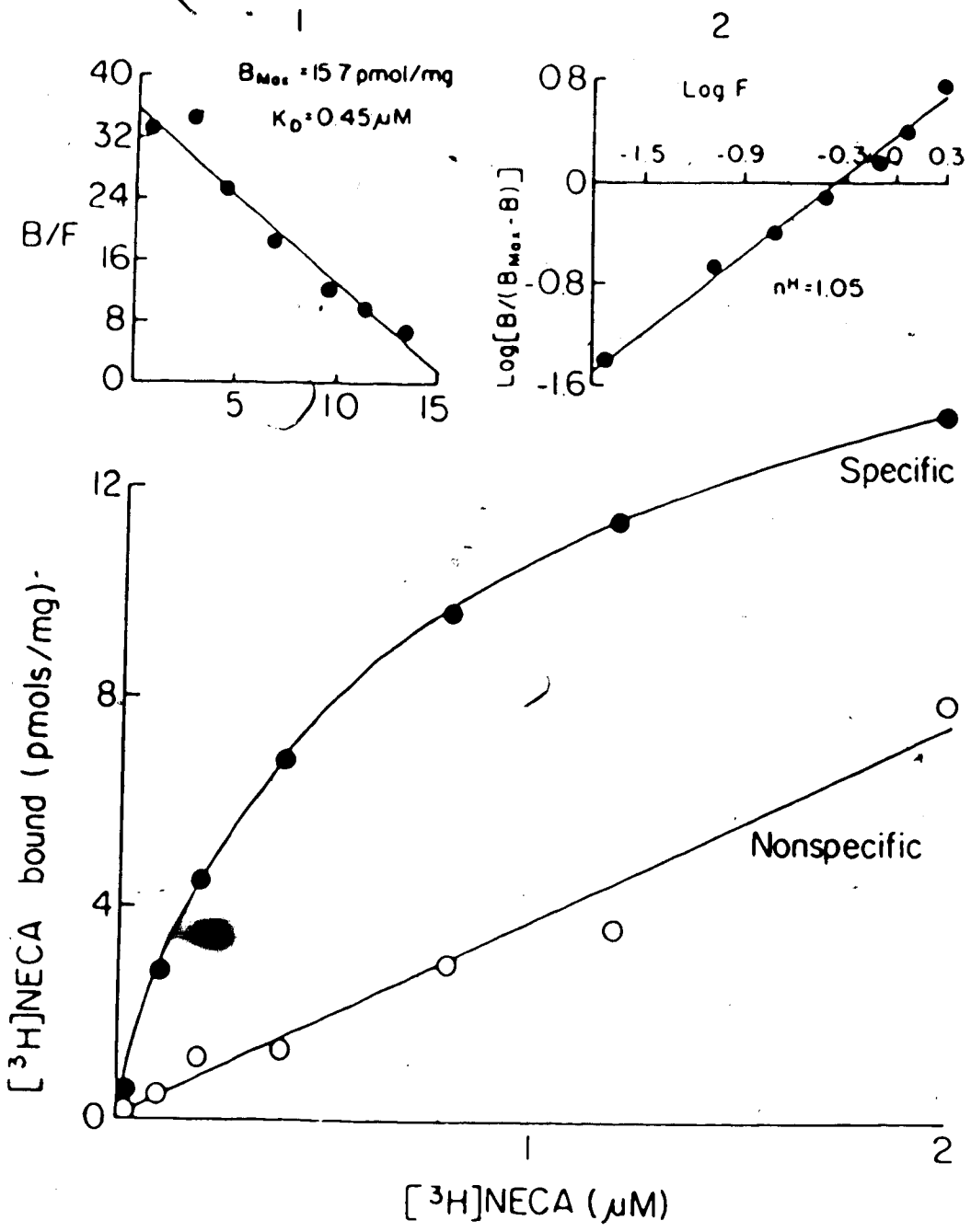


Fig. 26. Time course of association and dissociation of the specific binding of [^{3}H]NECA to longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with 20 nM radioligand at 2°C. 450-μl aliquots were withdrawn at various times (abscissa) up to 30 min and filtered as described under section 2.5.4.6. Dissociation was induced by adding NECA to the incubation at a final concentration of 100 μM. The inset shows a pseudo-first order plot of the association reaction which revealed a forward rate constant, $k_1 = 6.3 \times 10^5\ M^{-1}\ min^{-1}$. Values are mean of duplicate determinations with standard deviations of 5 - 11% at most points. Similar results were obtained in another assay.

Fig. 27 Concentration dependence of [^{3}H]NECA binding to longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with increasing concentrations of the radioligand at 2°C, as described under section 2.5.4.7. 500 μM NECA was used to estimate the nonspecific binding. Inset: The specific binding, B data was linearized on Scatchard (1) and Hill (2) plots. The slope of the Scatchard plot (r=0.97) provided an estimate for the affinity (K_D=0.45 μM) and B_{max} (15.7 pmols/mg). The slope of the Hill plot provided an estimate for the Hill coefficient (n^H=1.05). F is the free radioligand concentration. Values are means from two assays, each performed in triplicate. Standard deviations ranged between 8 - 15% at most points.


1
2
B_{Max} = 15.7 pmol/mg
K_D = 0.45 μM
B/F
40
32
24
16
8
0
5
10
15
Log F
0.8
0
-0.8
-1.6
-1.5
-0.9
-0.3
0
0.3
Log[B/(B_{Max} - B)]
n^H = 1.05
Specific
Nonspecific
12
8
4
0
1
2
[^{3}H]NECA bound (pmols/mg)
[^{3}H]NECA (μM)

respectively. On the other hand, the N^6-modified derivatives suc
R-PIA, S-PIA, CHA were poor inhibitors of [^{3}H]NECA binding (18 -
with the inhibitor at 200 μM concentration). Both IBMX
theophylline displaced the specific binding: IBMX (1 mM) by 92%
theophylline (5 mM) by 95%. By contrast, SPT was a very
inhibitor (less than 40% inhibition at 5 mM). The ribose-mod
adenosine derivatives which are known agonists at the intracellu
site also displaced [^{3}H]NECA binding effectively (Fig. 29).
binding was also sensitive to displacement by adenine.

Both L- and D-isomers of SAH displaced the specific bind
a Ki of 26.1 and 31.8 μM, respectively, as observed in a s
assay. ATP also inhibited the specific binding of [^{3}H]NECA and
inhibition was sensitive to adenosine deaminase (Fig. 30).
compounds such as inosine and Ro-20-1724 and calcium channel blc
such as nitrendipine and verapamil were weak inhibitors and di
achieve half maximal inhibition at the maximum concentration used
μM). Compounds like GTP and Gpp(NH)p and adenosine uptake blc
like dipyridamole and NBMPR were not effective at
8-Bromoadenosine was also not active at these sites (Table 7).
compounds which are antagonists at other receptor types su
haloperidol, methysergide, cimetidine, mepyramine, atropine
hexamethonium (all 100 μM) were inactive at [^{3}H]NECA binding
(data not shown).

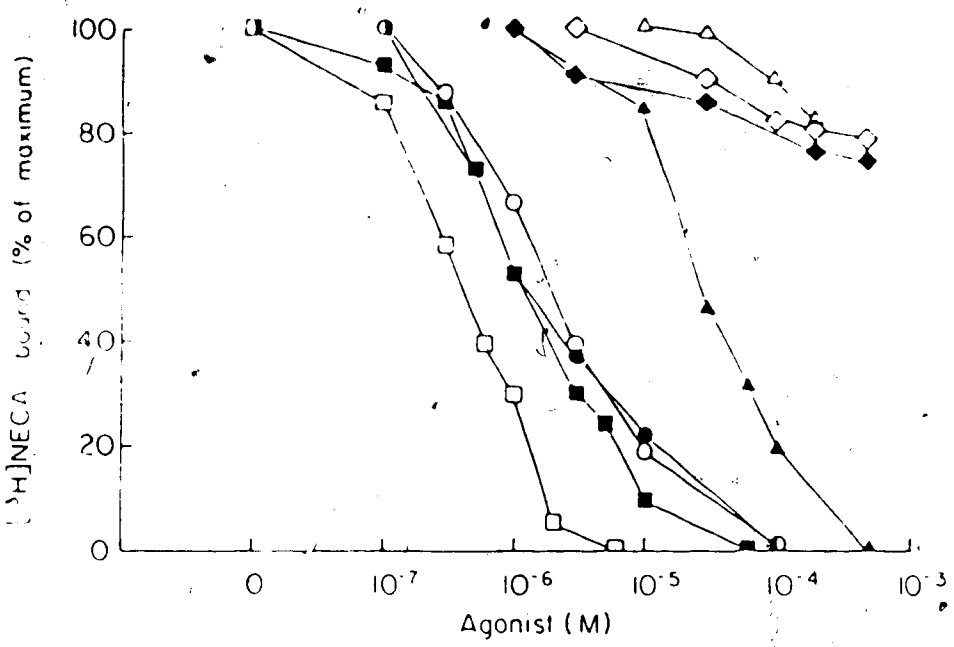


Fig. 28. Displacement of specifically bound [^{3}H]NECA by various agonists of adenosine receptors in longitudinal-muscle membranes of rabbit small intestine. The membranes were incubated with 20 nM radioligand at 2°C for 20 min in the presence of various concentrations (abscissa) of agonists: NECA (□), 2-chloroadenosine (■), MTA (●), CDA (○), adenosine (▲), S-PIA (◆), R-PIA (◇) and CHA (△). Values are means of triplicate determinations. Standard deviations at most points ranged between 5 - 12%.

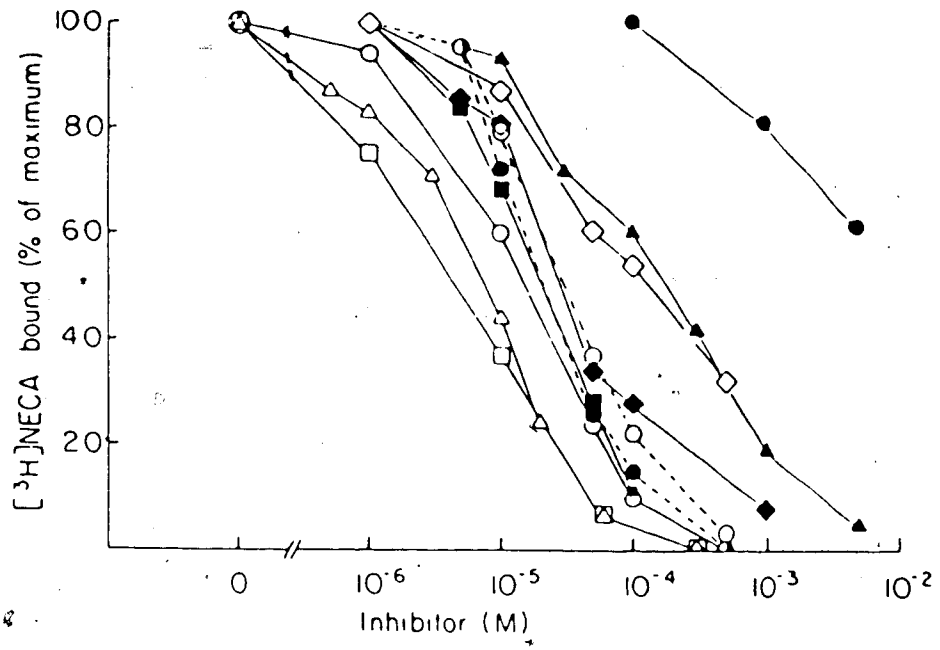


Fig. 29. Displacement of specifically bound [^{3}H]NECA by various inhibitors, in longitudinal-muscle membranes of rabbit small intestine. Membranes were incubated with 20 nM radioligand at 2°C for 20 min in the presence of various inhibitors: 3'-deoxyadenosine (□), 2':5'-dideoxyadenosine (△), 2'-deoxyadenosine (○), ATP (■), SAL-H (◆), SAD-H (○), IBMX (◆), adenine (◇), theophylline (▲) and SPT (●). Values are means of triplicate determinations with standard deviations between 4 - 12% at most points.

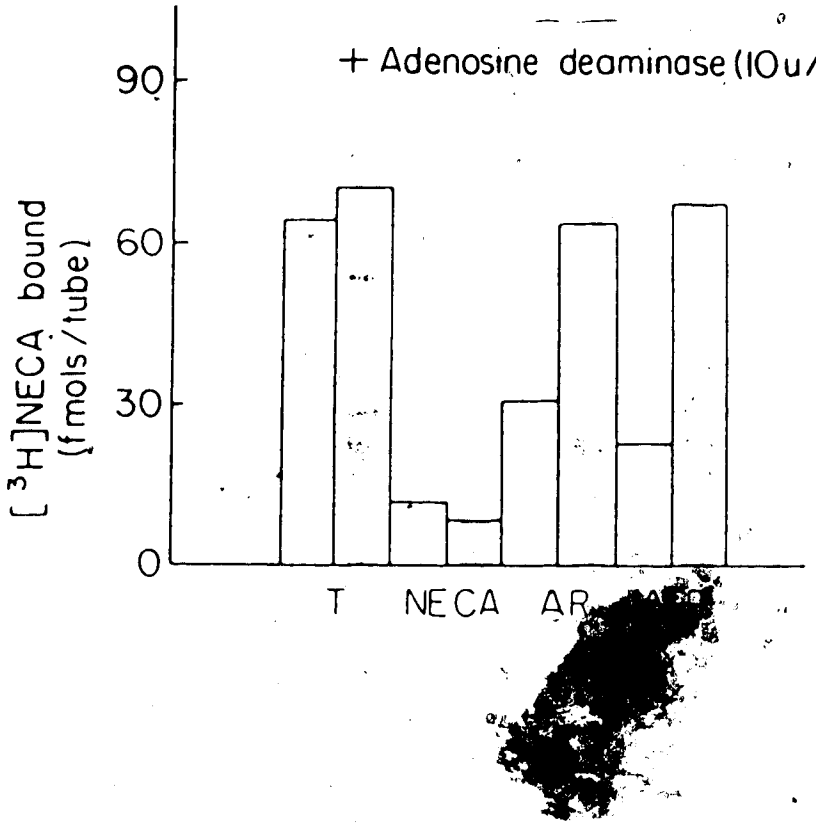


Fig. 30. Effect of adenosine deaminase on the displacement of [^{3}H]NECA binding by ATP in longitudinal-muscle membranes of rabbit small intestine. The membranes were first incubated at 37°C for 20 min in the absence and presence (shaded) of 10 U/ml adenosine deaminase. The incubations were subsequently transferred to 2°C for 5 min and filtered. Values are mean of triplicate determinations, with standard deviations of less than 11% (T is Total bound; AR is adenosine).

Table 7. Comparison of the inhibitory constant (Ki)* of various inhibitors of [^{3}H]NECA binding to rabbit intestinal membranes

Inhibitor	Ki (μM)	n^H	n
NECA	0.48 ± 0.04	1.20 ± 0.2	3
2-chloroadenosine	0.91 ± 0.02	1.09 ± 0.12	3
MTA	1.9 ± 0.13	1.05 ± 0.18	2
CDA	2.0	0.98	1
3'-Deoxyadenosine	3.7	0.93	1
2',5'-Dideoxyadenosine	4.4 ± 0.9	0.98 ± 0.05	2
2'-Deoxyadenosine	7.7	0.97	1
Adenosine	17.0 ± 5.3	0.85 ± 0.09	3
ATP	19.3	1.2	1
SAL-H	26.1	1.49	1
IBMX	29.1 ± 3.3	0.98 ± 0.02	2
SAD-H	31.8	1.35	1
Adenine	109.0	0.42	1
Theophylline	232.0 ± 48	0.51 ± 0.01	2

Inhibitor	Percent Inhibition**	n
CHA	24.7 ± 6.0 (100)	3
R-PIA	24.7 ± 2.9 (100)	3
S-PIA	23.0 ± 2.0 (100)	2
SPT	19.0 ± 0.9 (100)	3
Inosine	28.4 ± 3.0 (100)	2
RO-20-1724	15.0 (50)	1
Dipyridamole	- (100)	1
NBMPR	- (100)	1
Nitrendipine	29.1 ± 9.7 (100)	2
Verapamil	4.0 ± 1.2 (100)	2
GTP	- (100)	2
Gpp(NH)p	- (100)	2

The value of the regression coefficient r (Hill plots) ranged between 0.97 and 0.997.

*The Ki values were estimated using the equation provided by Cheng and Prusoff (1973), as described under sec. 2.8.1.1.3.

**Numbers in parentheses represent the concentrations in μM at which the corresponding percent inhibition was observed.

The Hill coefficients estimated for all agonists used as inhibitors of binding were close to unity (0.85 - 1.2). On the other hand, theophylline displaced the binding with a considerably lower Hill coefficient value (n^H=0.51).

3.5 Effect of nucleosides on the spontaneous contractility of rabbit small intestine

NECA, CDA and MTA produced dose-dependent relaxation of the isolated intestinal tissue. NECA was the most potent relaxant (ED_{50}=0.11 nM) followed by CDA (ED_{50}=1.2 μM). MTA was a weak but full agonist with an ED_{50} = 110 μM (Fig. 31, Table 8). The relaxations were competitvely antagonized by SPT and the ED_{50} values in the presence of SPT were 3.1 μM for NECA, 77 μM for CDA and 225 μM for MTA (Table 8). These values of ED_{50} for all three agonists were found significantly different (Student's t-test for paired data) at $p<0.05$).

3.6 Effect of NECA and MTA on the adenylate cyclase activity in mouse neuroblastoma cell membranes.

NECA stimulated the basal adenylate cyclase activity (8.5 - 10 pmols/mg/min) by 134% at a 10 μM concentration, with an ED_{50} of 0.5 μM (0.14 - 1.4, 95% confidence limits). By contrast, MTA did not affect the basal enzyme activity up to a concentration of 100 μM tested. However, at the same concentration, MTA shifted the dose response curve for NECA to the right, in a competitive fashion, with an ED_{50} for NECA = 12.0 μM (1.3 - 112, 95% confidence limits, Fig. 32). In a Student's t-test for paired data, these values of ED_{50} were found to

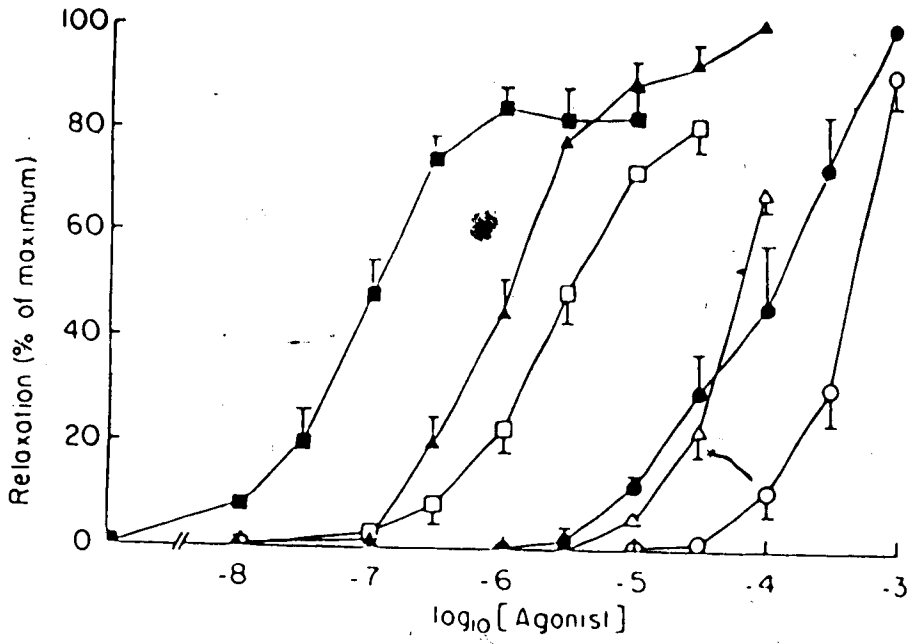


Fig. 31. Effect of some 5'-derivatives of adenosine on the spontaneous contractility of isolated small intestine from rabbit. Tissues were isolated and tested as described under section 2.6. 100 μM SPT was used to study the antagonism of responses. (■) NECA alone and in the presence (□) of SPT. (▲) CDA alone and in the presence (△) of SPT. (●) MTA alone and in the presence (○) of SPT. Each curve represents means ± S.E.M. from 4 different experiments.

Table 8. Effect of adenosine derivatives on the inhibition of spontaneous contractility of isolated small intestine from rabbit[1]

Compounds	ED_{50}	95% Confidence Limit	Antagonism by 100 µM SPT[3]	n
Adenosine[1,2]	5.5	2.3-14	+	5
2-Chloroadenosine[1]	0.51	0.08-3.6	+	5
R-PIA[1]	0.023	0.15-0.035	+	5
R-HPIA[1]	0.064	0.024-0.18	+	5
S-PIA[1]	1.0	0.12-8.5	+	4
NECA[1]	0.11	0.06-0.21	+	4
CDA	1.2	0.9-1.5	+	4
MTA	110.0	52-230	+	4
Adenine	>300			2
Inosine[1]	>300			2
3'-Deoxyadenosine[4]	≈100 µM		(+)	2
2'-Deoxyadenosine[4]	NA			2
2',5'-Dideoxyadenosine[4]	NA			2
SAL-H[4]	NA			2

[1] data partly from Baer and Vriend (1985)
[2] in the presence of 1 µM HNBTG
[3] significant difference in EC_{50} values (at $p<0.05$)
[4] tested at a single concentration of 100 µM

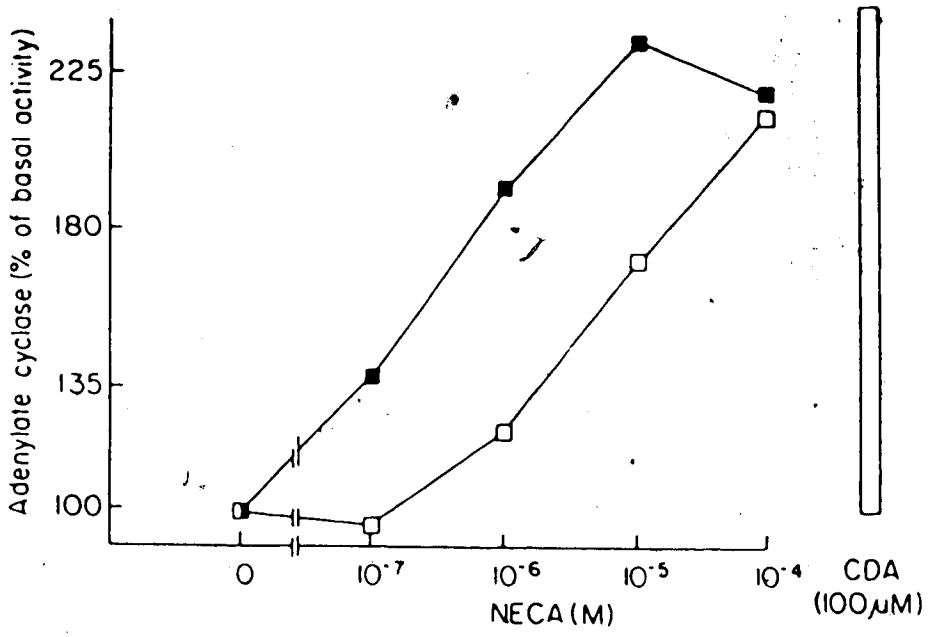


Fig. 32. NECA-induced stimulation of the basal adenylate cyclase activity in neuroblastoma cell membranes and its antagonism by MTA. Adenylate cyclase activity was measured at 37°C for 20 min. (■) NECA alone and in the presence (□) of 100 μM MTA. Values are means from 3 different assays each performed in triplicate. The S.E.M.s were below 15%. The bar represents the mean of stimulation of the basal enzyme activity by 100 μM CDA from two assays each performed in triplicate. The standard deviation was 9%.

be significantly different at $p<0.05$. 5'-chloro-5'- deoxyadenosine (CDA), which was tested at a single concentration of 100 μM, produced a 144% increase in the enzyme activity over basal (Fig. 32).

4. DISCUSSION

4. DISCUSSION

4.1 Development of the radioligand of high specific activity

Adenosine receptors in various systems such as brain, fat cells, liver, heart, platelets and some smooth muscles have been identified using tritium-labelled adenosine agonists such as 2-chloroadenosine, R-PIA, CHA, and NECA and the antagonist DPX (Daly, 1982; 1983; 1985; Williams, 1983; Schwabe, 1981, 1983, 1985). Tritium-labelled ligands suffer from the inherent problem of low specific radioactivity. Identification of receptors by radioligand binding is much facilitated by the use of labels such as ^{125}I which offer the advantage of high specific radioactivity. This is particularly true for systems where the density of receptors may be low or where the availability of the tissue material is limited as in the case of cultured cells. Furthermore, owing to their low specific radioactivity, the use of [^{3}H]-labelled ligands is limited to systems with high receptor density and with a dissociation constant of ligand-receptor complex of about 0.1 nM or more. For these reasons, [^{125}I]-labelled ligands with high specific radioactivity have been developed for various receptor systems: for example, [^{125}I]iodohydroxybenzylpindolol for beta-adrenergic receptors(Aurbach et al., 1974), [^{125}I]bungarotoxin for nicotinic cholinergic receptors (Conti-Tronconi and Raftery, 1982), and [^{125}I]-labelled peptides for certain peptide receptors such as [^{125}I]-labelled vasoactive intestinal polypeptide (Suzuki et al., 1985). Development of an [^{125}I]-labelled adenosine derivative was therefore undertaken to investigate the receptors in rabbit gut. It

was assumed that the new radioligand would allow measurement of adenosine receptors even if the density of the receptors in the system was very low.

It had been shown earlier that R/S-HPIA stimulated adenylate cyclase activity in murine neuroblastoma cell membranes and decreased the spontaneous activity of isolated small intestine from rabbit with about a 10-fold lower potency than R-PIA (Baer, unpublished results). The phenolic hydroxy group in HPIA renders the compound amenable to iodination. Preliminary data using the iodinated compound R/S-IHPIA revealed that its potency was similar to that of R/S-HPIA in adenylate-cyclase systems in neuroblastoma and isolated rabbit small intestine (data not shown). Furthermore, when R/S-HPIA was radioiodinated, the preliminary data from binding studies utilizing [^{125}I]R/S-IHPIA and rat brain membranes seemed to satisfy the criteria expected for ligand binding to adenosine receptors (Munshi and Baer, 1982; Schwabe et al., 1982). Since HPIA is an N^6-substituted derivative with a structure similar to that of PIA, it was expected that both HPIA and IHPIA would have high affinity at the classical A_1 receptors, such as occur in brain and fat cells. It was also expected by analogy with results obtained with the enantiomers of PIA that IHPIA would show steroselective binding characteristics, with the R-enantiomer of IHPIA having higher affinity at adenosine receptors.

Accordingly, R-HPIA was synthesized and radioiodinated. The interaction of the new radioligand, [^{125}I]R-IHPIA, with rat brain adenosine receptors was studied. The use of PEI TLC was particularly

effective in separating carrier-free [^{125}I]R-IHPIA from the unlabelled R-HPIA, yielding a pure ligand. PEI TLC is an anion-exchange chromatographic system that makes use of the fact that the acidity of the phenolic hydroxy group is increased by mono- and di-iodination. It is applicable to other iodinated derivatives of compounds of small molecular weight, as has been recently shown in this laboratory with the radio-iodination of cyclic nucleotide derivatives (Schmidt and Baer, 1984).

4.2 Characteristics of the interaction of R-IHPIA with brain adenosine receptors

Since adenosine derivatives inhibit the activity of the enzyme adenylate cyclase in various regions of brain, the similar effect produced by R-IHPIA was studied utilizing cerebellar adenylate cyclase. The basal activity of the enzyme in rat cerebellar membranes is high (Prentki et al., 1979; Wolpik et al., 1985), exceeding that in some other regions of brain. For example, adenosine receptor-mediated inhibition of the adenylate cyclase has been reported in cortex (Cooper et al., 1980), striatum and hippocampus (Yeung and Green, 1984). GTPγS-induced inhibition of cerebellar adenylate cyclase (Fig. 3) was used to demonstrate that the cerebellar membrane Ni unit was functional under the assay conditions. Cooper and Londos (1979) showed that measurement of the effects of adenosine and its derivatives on adenylate cyclase is greatly facilitated by reducing the concentration of endogenous adenosine, formed from ATP, in the assay medium by substituting dATP for ATP as the substrate. A dATP

assay system was therefore used.

Adenosine receptor-mediated inhibition of adenylate cyclase activity is strictly dependent on the presence of GTP (Londos et al., 1978). GTP concentrations of 4 - 100 μM have been used in various systems (Londos et al., 1978; Cooper et al., 1980; Yeung and Green, 1984). Furthermore, Na^+ has been shown to increase adenosine receptor mediated inhibition of adenylate cyclase activity in various systems such as brain (Cooper et al., 1979; Yeung and Green, 1984) and fat cells (Londos et al., 1981). The precise mechanism of the Na^+ effect is not known. Cooper et al. (1980) suggested that Na^+, which stimulates basal enzyme activity on its own, amplifies the hormonal inhibition by reversing GTP-induced inhibition of the enzyme activity. Our preliminary results (data not shown) revealed that GTP (0.1 - 100 μM) and Na^+ (20 - 140 mM) both inhibited basal enzyme activity in the cerebellar membranes. Although inhibition by Na^+ was less in the presence of GTP (0.1 - 100 μM), Na^+ did not reverse the GTP-induced inhibition as was the case in rat cerebral cortical membranes (Cooper et al., 1980). This lack of effect of Na^+ on GTP-induced inhibition of adenylate cyclase activity is not unique to the rat cerebellar membrane system, since it has also been demonstrated with adenylate cyclase in human platelet membranes (Steer and Wood, 1981). Sodium was, therefore, omitted from the assay for the study of the inhibitory effects of R-PIA and R-IHPIA on cerebellar adenylate cyclase.

In a single assay, the potency of R-IHPIA in the cerebral adenylate cyclase system was found quite similar to that of R-PIA and

both had comparable efficacies. Recently, the activity of R-IHPIA as an agonist ligand for A_1 receptors has been demonstrated. Linden (1984) demonstrated similar potency (about 10 nM) and efficacy of R-PIA and R-IHPIA for the inhibition of isoproterenol-stimulated cAMP accumulation in rat fat-cell ghosts. This author also demonstrated comparable affinities of R-PIA and R-IHPIA on isolated electrically driven left atria of rat; in that study, the inhibition of developed tension was competitively antagonized by theophylline. Ukena et al. (1984a) have demonstrated a 4- to 6-fold lower potency of R-IHPIA (IC_{50}=60 nM) relative to R-PIA (IC_{50}=16 nM) in (i) the inhibition of the adenylate cyclase system of fat cells from rat and (ii) inhibition of adenosine deaminase-stimulated lipolysis. Thus, the biological activity of R-IHPIA on A_1-receptors is demonstrable in a variety of systems, with potency comparable to that of R-PIA.

Characterization of binding sites of [^{125}I]R-IHPIA was attempted to study the latter's utility as a radioligand for adenosine receptors using rat brain membranes as the model system. [^{3}H]R-PIA had earlier been used to characterize adenosine receptors in rat brain membranes (Schwabe and Trost, 1980) and a similar membrane preparation was used to characterize binding of [^{125}I]R-IHPIA. The initial experiments revealed that [^{125}I]R-IHPIA was not metabolized under the conditions of incubation (section 3.3.1) and that the radioactivity bound to the brain membranes was completely recovered as [^{125}I]R-IHPIA (section 3.3.2). Furthermore, the radioligand binding to the glass-fibre filter papers was less than 0.1% and not displaceable with the excess unlabelled ligand (R-PIA).

[^{125}I]R-IHPIA binding to rat brain membranes was dependent on the protein concentration (Fig. 5) and temperature (Fig. 6). The effect of pH on the binding (Fig. 7) may possibly be explained as follows. The acidity of the phenolic hydroxy group is increased upon iodination such as in the case of tyrosine (Bolton, 1977). This could be presumed to lower the pKa of IHPIA relative to HPIA and permit IHPIA to ionize in a medium with a basic pH. As [^{3}H]R-PIA binding to the same membrane preparation appears not to differ over a range of pH (Fig. 7B), the loss of specific binding of [^{125}I]R-IHPIA at pH 8 may be due to a significant ionization of the molecule at higher pH. Since pH and temperature dependence of the binding were studied at concentrations of [^{125}I]R-IHPIA much below its K_D, the lower binding of the radioligand at higher pH or at higher temperature may be a result of decreased affinity and/or decreased number of sites for the radioligand.

We found the kinetics of the association reaction to be slow (Fig. 8). The value of the forward rate constant (k_1=7.5 x 10^6 M^{-1} min^{-1}) is similar to that reported for [^{3}H]R-PIA binding (k_1=6.58 x 10^6 M^{-1} min^{-1}, at 37°C) to rat brain membranes (Schwabe and Trost, 1980) and rat fat-cell membranes (k_1=2.6 x 10^7 M^{-1} min^{-1}, at 37°C) (Ukena et al., 1984a) and for [^{125}I]R-IHPIA binding (k_1=2.55 x 10^7 M^{-1} min^{-1}, at 21°C) also to rat brain membranes (Linden, 1984). The relatively slow association kinetics may be due to physico-chemical properties of these ligands that cause their interaction with the receptors to be slow. Alternatively, the slow association may be due to a slow interaction between the ligand bound receptor and Ni.

The kinetics of dissociation of the bound radioligand was biphasic (Fig. 9). A similar dissociation pattern has been reported using other radioligands for adenosine receptors such as [^{3}H]R-PIA binding to rat brain membranes (Schwabe and Trost, 1980), [^{3}H]CHA binding to guinea-pig brain membranes (Goodman et al., 1982) and [^{3}H]NECA binding to A_2 receptors in rat striatal membranes (Yeung and Green, 1984). Non-linear first-order dissociation plots could result either from the presence of heterogenous binding sites (or ligands) or from co-operative site-site interactions.

Biphasic dissociation curves have been demonstrated for radioligand binding to other receptor systems such as [^{3}H]GABA binding to mammalian brains (Olson et al., 1981) and [^{3}H]naltrexone binding to rat brain membranes (Fischel and Medzihradsky, 1981). In each case, the Scatchard analysis also revealed the presence of two sites differing in affinity by at least 20-fold. Although both A_1 and A_2 adenosine receptors have been reported in brain, the high affinity (K_D around 2 nM) of [^{125}I]R-IHPIA (Fig. 10) coupled to the stereospecific displacement by PIA and HPIA (Fig. 11) indicate that the binding is to A_1 receptors. Also, the Scatchard analysis revealed a linear plot suggestive of binding to a single population of sites (Fig. 10). Furthermore, the Hill plots from saturation and displacement (R-IHPIA) binding data did not reveal co-operativity in the interaction (Fig. 10 and Table 4). A linear Scatchard plot could result either from a less than 10-fold difference in the K_D value for the two sites or from a small proportion of one of the sites relative to the total number of sites. However, there would probably

be no appreciable binding of [^{125}I]R-IHPIA to A_2 sites at the maximum concentration of 35 nM used in the saturation assays and particularly in dissociation assays where a low radioligand concentration of 0.48 nM was used. Thus the biphasic dissociation of [^{125}I]R-IHPIA does not appear to result from binding to both A_1 and A_2 receptors or from co-operative site-site interactions in binding to A_1 receptors.

Using low concentrations (up to 5 nM) of [^{125}I]R-IHPIA in saturation assays, Stiles (1985) has demonstrated high-affinity binding of the radioligand to cerebral cortical membranes from rats, both in the absence and presence of Gpp(NH)p (B_{max} 50% of that in the absence of Gpp(NH)p, but the same K_D). This suggests that the adenosine A_1 receptor is more tightly coupled to Ni, at least in this system, than are other receptor types in various systems such as the alpha$_2$-adrenergic (Smith et al., 1981; Michel et al., 1981), beta-adrenergic (Limbird and Lefkowitz, 1978), or dopamine D_2 receptors (De Lean et al., 1982), where no high-affinity binding of the ligands is observed in the presence of guanine nucleotides. A tight coupling of A_1 - Ni is further demonstrated by the observation that guanine nucleotide sensitivity is retained in solubilized brain adenosine receptor preparations (Gavish et al., 1982; Stiles, 1985; unpublished data from our lab). Furthermore, the sedimentation properties of the guanine nucleotide-sensitive A_1 - Ni complex are the same regardless of whether the receptor is prelabelled or not. On the basis of this evidence, Stiles (1985) has suggested that a certain proportion of the A_1 receptors must be intimately associated with Ni even without agonist occupation of the receptor and would therefore be less

sensitive to dissociation by guanine nucleotides in membranes, thus accounting for the high affinity binding of [^{125}I]R-IHPIA in the presence of Gpp(NH)p. Because in Stiles' experiments about 50% of the high-affinity binding was insensitive to Gpp(NH)p, about half of the population of A_1 receptors may be intimately associated with Ni under the incubation conditions.

In the light of Stiles' observations, it is conceivable that the slower phase of the dissociation of [^{125}I]R-IHPIA (Fig. 9) corresponds to A_1 sites of very high affinity, which results from a tighter coupling of a proportion of A_1 receptors with Ni, which would remain undetected in saturation assays for the reasons discussed above. The persistence of a slower phase of dissociation in the presence of Gpp(NH)p (Fig. 9) substantiates this idea. Goodman et al. (1982) have also reported two phases of dissociation of radioligand specifically bound to brain adenosine receptors in the presence of guanine nucleotides.

The value of the dissociation rate constant (k_2=0.018 min^{-1}) obtained is similar to that reported by Linden (1984) (k_2=0.024 min^{-1}), using [^{125}I]R-IHPIA and rat brain membranes. The line in the inset 2 (Fig. 9) does not pass through zero on the y-axis. This is because of a quick, initial dissociation of about 20% of the specifically bound radioligand during the first 3 min. It was, however, not possible to quantitate this phase of dissociation because filtration assay would not allow measurement of dissociation at lower time intervals. It is possible that this initial rapid phase of dissociation is due to the presence of some endogenous GTP in the membrane

preparation which would convert a proportion of the high affinity receptors in the low affinity state (see 1.4.5). The bound radioligand would then dissociate rapidly from these lower-affinity sites. The dissociation constant (K_D) of the R-IHPIA- receptor complex as derived from the kinetic and saturation analyses, 2.4 nM (Fig. 9) and 1.85 nM (Fig. 10), respectively, are in good agreement. Similar K_D values (Table 9) for [^{125}I]R-IHPIA in rat brain membranes have been reported by Linden (1984) and Stiles (1985).

Displacement binding data from a single assay showed that addition of a hydroxy group at carbon 4 in the phenyl ring of PIA does not change the affinity of the compound for the receptors (Fig. 11). Further addition of an iodo-group (at carbon 3 or 5 in the phenyl ring) apparently decreases the affinity slightly; R-IHPIA being slightly less potent than R-PIA in our assay (Fig. 11 and Table 4). Specifically bound [^{125}I]R-IHPIA is also displaced by other agonists and antagonists of adenosine receptors. Although these results were obtained in single assays, the Ki values for various agonists (Table 4) are comparable to those reported for [^{125}I]R-IHPIA binding to rat brain membranes (Linden, 1984), rat fat-cell membranes (Ukena et al., 1984a) and solubilized rat brain receptors (Stiles, 1985). Similarly, a 12-fold difference in the affinity of stereoisomers of PIA in our assay is comparable to what has been reported in systems described above.

As previously discussed (Introduction), the rank order of potency of various agonists is displacement of bound [^{125}I]R-IHPIA from the

Table 9. [^{125}I]R-IHPIA binding to membranes from rat brain, heart and fat cells

System	Temperatures (°C)	pH	K_D (nM)	B_{max} (fmols/mg)	Reference
Rat Brain	21	7.9	1.94 ± 0.74	871 ± 31	Linden, 1984
	37	7.4	0.7 ± 0.1	770 ± 20	Stiles, 1985
	30	7.4	2.1	1040	(Our studies)
Rat Heart	21	7.9	3.11 ± 0.95	22 ± 8	Linden, 1984
Rat Fat Cells	21	7.9	2.95 ± 0.84	379 ± 11	Linden, 1984
	37	7.4	0.7, 7.6	940, 950	Ukena et al., 1984

rat brain receptor preparation (Fig. 11), together with a more than 10-fold difference in the potency of R- and S-PIA, suggests that [^{125}I]R-IHPIA was binding to A_1-receptors (sec. 1.3.6.3). The observed 11-fold higher Ki for NECA as compared to that for 5'-N-methylcarboxamidoadenosine (MECA) is similar to the difference in their Ki values reported in rat fat cell membranes for [^{3}H]R-PIA binding (Ukena et al., 1984b) and is consistent with binding to A_1 receptors. For most of the agonists tested in the present study, the Hill coefficient values were around 0.8 (Table 4). Although this does not represent a significant deviation from a slope of unity, the existence of more than one affinity state (see above) may account for the relatively low values of the Hill coefficient for agonist displacement curves.

Bound [^{125}I]R-IHPIA is displaced from rat brain adenosine receptors by compounds that are known agonists or antagonists at adenosine receptors (Fig. 11, 12). Several compounds that are not known to have an affinity for adenosine receptors did not compete for the binding of [^{125}I]R-IHPIA (Fig. 22). Thus, the characteristics of [^{125}I]R-IHPIA binding to brain adenosine receptors seem to satisfy most criteria for radioligand binding to high affinity adenosine receptors. The binding is dependent upon protein concentration (Fig. 5) and temperature (Fig. 6) and meets the specificity criteria required for binding to A_1 adenosine receptors as well as being sensitive to displacement by the xanthine antagonists. Furthermore, the influence of Gpp(NH)p on the dissociation reaction (faster phase) suggests that the displaceable binding is related to adenylate cyclase

suggests that the displaceable binding is related to adenylate cyclase-coupled receptors. However, the slow establishment of equilibrium in the binding of [^{125}I]R-IHPIA may be a potential problem in some systems. A 2-h long incubation may entail loss of receptor functions because of proteolytic enzyme action. On the other hand, slow dissociation rates minimize artefacts in binding assays caused by dissociation of the ligand-receptor complex during the processes of separation and washing.

[^{125}I]R-IHPIA has already been used to characterize adenosine receptors in systems other than brain. Ukena et al. (1984a) have demonstrated the presence of high affinity binding sites (K_D=0.7 nM) in rat fat-cell membranes, although such sites were not evident in an identical membrane preparation using [^{3}H]R-PIA as the radioligand (Trost and Schwabe, 1981). However, Linden (1984) reported problems with the use of [^{125}I]R-IHPIA in rat heart ventricular membranes particularly with respect to a high ratio of nonspecific to specific binding of this radioligand. It therefore remained to be seen whether [^{125}I]R-IHPIA would prove a useful radioligand for characterizing adenosine receptor in systems other than brain and fat cells. Our experiments have shown that, contrary to what is observed in brain membranes, [^{125}I]R-IHPIA binds to small-intestinal membranes from rabbit with a high component of nonspecific binding, with very rapid kinetics and without specificities that define classical adenosine receptors.

4.3 Characteristics of adenosine receptor in intestinal smooth muscle from rabbit

The potency of R-HPIA for the relaxation of isolated rabbit small intestine is similar to that of R-PIA (Baer and Vriend, 1985; Table 8). Preliminary data revealed that R/S-IHPIA produced the intestinal relaxation with approximately 30-fold less potency than R-HPIA (data not shown). Given an approximately 40-fold difference in the potency of the stereoisomers of PIA in this system (Table 8), one would expect R-IHPIA to approximate the potency of R-PIA and R-HPIA. Characterization of adenosine receptors from rabbit intestine was therefore attempted using [^{125}I]R-IHPIA and, as a means of comparison, using [^{3}H]R-PIA.

4.3.1 [^{125}I]R-IHPIA- and [^{3}H]R-PIA-binding sites in the intestinal membranes

As described above, the nonspecific binding of both radioligands was very high and accounted for 50 - 70% of the total binding of [^{125}I]R-IHPIA and 40 - 65% of the total binding of [^{3}H]R-PIA in various experiments. Preliminary data revealed that the binding of each radioligand was displaceable only by N^6-derivatives such as PIA and CHA. Compounds such as NECA and 2-chloroadenosine were not effective at these sites. Furthermore SPT, which antagonizes the relaxant response of PIA, NECA, and 2-chloroadenosine in the isolated intestinal preparation (Table 8), did not displace the binding of either radioligand. Further studies of the binding specificity revealed that certain compounds that are known antagonists of other

receptor types such as dopamine, serotonin, and histamine-H_1 and -H_2 displaced the bound radioligands from intestinal membranes (Fig. 21). However, these compounds had no appreciable effect on [^{125}I]R-IHPIA binding to rat brain membranes (Fig. 21).

One of the reasons for the anomalous nature of radioligand binding to functional receptor sites may be degradation or enzymatic modification of the radioligand under the conditions of incubation. However, extraction of the bound radioactivity and subsequent autoradiography (sec. 3.4.2.1) revealed that this was not the case with [^{125}I]R-IHPIA. Radioligand binding to adenosine receptors in various systems is known to be dependent on pretreatment of the membranes with adenosine deaminase (see review byDaly, 1982) to deplete endogenous adenosine. However, pretreatment of the intestinal membranes with the enzyme did not increase the amount of the bound radioligand (Fig. 15). Preliminary experiments that were performed in the presence of adenosine deaminase (5 U/ml) also revealed poor affinity of NECA at [^{125}I]R-IHPIA and [^{3}H]R-PIA binding sites and vice versa (see also below).

A clue to the problem with the use of these radioligands was provided by our studies of the kinetics of their binding to the intestinal muscle membranes. The rapid dissociation of the bound radioligand is an indication that the receptor-specific binding is lost during separation of the bound and free radioligand by filtration.

Anomalous specificity of radioligand binding to adenosine receptors has also been reported in other systems. [^{3}H]R-PIA

(10 nM) binds to human platelet membranes with a 45% nonspecific binding component (in the presence of 1 mM R-PIA) and the binding is not displaced by NECA (Huttermann et al., 1984), although both PIA and NECA stimulate the adenylate cyclase activity of platelets with only a 5-fold different potency. [^{3}H]CHA binding to rat liver plasma membranes is poorly displaced by NECA, although CHA is only about 10-fold less potent than NECA in adenylate cyclase assays in similar membrane preparations (Schutz et al., 1982). [^{3}H]CHA binding in this system (liver) is sensitive to displacement by 2',5'-dideoxyadenosine which is about 5-fold more potent than NECA. Furthermore, in a recent report, no detectable [^{3}H]CHA binding was observed in membranes from human uterus (Ronca-Testoni et al., 1984). It thus appears that, unlike as in brain membranes, radiolabelled-N^6-derivatives of adenosine may not allow measurement of adenosine receptors in some systems such as platelet and liver membranes.

4.3.2 [^{3}H]NECA binding sites in the intestinal membranes

NECA decreases the spontaneous activity of isolated small intestine from rabbit (Table 8, Baer and Vriend, 1985) with an ED_{50} of 110 nM, about 3-fold less potent than R-PIA. After this unsuccessful attempt to label the smooth muscle adenosine receptor with radio-labelled N^6-derivaties of adenosine, [^{3}H]NECA was used to further the investigation. [^{3}H]NECA binding to intestinal membranes was rapid and rapidly reversible (Fig. 26). Nonspecific binding of this radioligand was low (24% of the total binding, at K_D) and there was no detectable binding to the glass-fibre filters. As observed with

labelled N^6-derivatives, the binding of [^{3}H]NECA was not sensitive to pretreatment with adenosine deaminase (Fig. 22).

[^{3}H]NECA binding was membrane concentration (Fig. 23) and temperature dependent (Fig. 24). The finding that measureable binding is highest at low temperature (2°C) may be explained by the high rate of dissociation of the bound ligand (Fig. 26). The decrease in specific binding with higher temperatures does not result from metabolism of the radioligand to products with lower binding affinity, as incubation at 37°C for the standard incubation time of 30 min and subsequent cooling of samples to 2°C before filtration did not appreciably reduce the amount of [^{3}H]NECA binding (data not shown).

[^{3}H]NECA bound to human platelet membranes (Huttemann et al., 1984), and to membranes from rat brain microvessels (Schutz et al., 1982), and [^{3}H]2-chloroadenosine bound to human placental membranes (Fox and Kurpis, 1983) exhibit similar rapid dissociation kinetics; in those studies, the bound and free radioligand were separated in a filtration assays using 2 washes of 4 or 5 ml each. However, the rapid dissociation of the bound radioligand at equilibrium would not allow measurement of the receptor-specific binding.

Mass law analysis of the equilibrium binding data revealed that, [^{3}H]NECA binds a single class of saturable sites (K_D=0.45 μM, Fig. 27), the maximum number of sites bound was very high (16.9 pmols/mg protein retained on the filter paper) relative to that reported in brain (Yeung and Green, 1984). [^{3}H]NECA binding sites in the intestinal membranes differ from those in striatal membranes for both K_D and B_{max} (Table 10), affinity in the intestinal membranes being

more than 25-fold lower than in the brain membranes, although comparable to that at the high-affinity sites, in other systems, such as in membranes from liver, platelets and uterus (Table 10,11). It appears, therefore, that [^{3}H]NECA binding sites in these systems are different from those in striatum which are known to be functional receptor sites. However, the apparent lack of adenosine receptor sites in some systems may be an artefact of the binding assay due to loss of the site specific binding during separation of the bound and free radioligand.

This inference is further substantiated by findings on the ~~specificty~~ of [^{3}H]NECA binding sites. Table 11 shows that there is only about a 5-fold difference in the potency of NECA and R-PIA at [^{3}H]NECA binding sites in rat striatum, although in the peripheral systems this difference is more than a 1000-fold. However, adenosine receptors in striatum, as well as in other systems, such as liver and platelets, are known to be linked to adenylate cyclase activity (Yeung and Green, 1984; Schutz et al., 1982; Huttermann et al., 1984). The difference in the potency of NECA and R-PIA for stimulating the enzyme is only about 5 - 10 fold in all three systems. This suggests that in the these systems [^{3}H]NECA binding sites may be different from those mediating adenylate cyclase stimulation. Effective displacement of [^{3}H]NECA from its binding sites by compounds such as adenine and 2',5'-dideoxyadenosine further substantiates this idea(Table 7). In intestinal smooth-muscle preparations, compounds such as adenine, 2'-deoxyadenosine, 3'-deoxyadenosine, 2',5'-didexoyadenosine and SAH were inactive (Table 8), although each displaced [^{3}H]NECA binding in

Table 10. Parameters of [^{3}H]NECA binding to membranes from various tissues

System	B_{max}*	K_{D1}**	B_{max}*$_2$	K_{D2}**	References
Rat liver	1.03	0.168	29.4	4.2	Schultz et al. (1982a)
Human platelets	8.4	0.160	33.4	2.9	Huttermann et al. (1984)
Human uterus	2.3	0.123	-	-	Ronca-Testoni et al. (1984)
Rabbit small intestine	16.9****	0.45	-	-	(These studies)
Rat brain microvessels***	0.084	0.019	5.2	2.5	Schultz et al. (1982b)
Rat striatum*** (NEM-pretreated)	0.188	0.017	-	-	Yeung and Green (1984)

*pmols/mg protein

** μM, at 0 - 4°C (25°C, rat brain microvessels)

***at 37°C

****B_{max} underestimated by 20%

Table 11. Specificity of [^{3}H]NECA binding in various systems*

Compounds	[1]Calf Thymocytes	[2]Human Platelets	[3]Human Uterus	[4]Rabbit Intestine	[5]Rat Striatum**
NECA	0.32	0.5	0.19	0.46	0.386
2-chloroadenosine	2	6	4	1.11	1.3
Adenosine	1400	>1000	-	17	-
R-PIA	>1000	>1000	240	>100	1.6
CHA	-	>1000	-	>100	2.1
S-PIA	>1000	>1000	-	>100	20.8
IBMX	32	92	110	29.1	-
Theophylline	325	770	-	232	60.5
2',5'-dideoxyadenosine	78	14	-	4.4	>100
Adenine	190	86	-	109	>100
Dipyridamole	>100	>100	-	>50	-
Inosine	>1000	>1000	-	>100	>100

*The numbers represent K_i values (μM)
**The number represents IC_{50} values (μM), The values for Ki would be 4.5 - 6.2 fold lower (based on K_D=17 nM and total radioligand concentration of 60 - 88 nM per incubation).
(1) Ukena et al., 1982; (2) Huttermann et al., 1984; (3) Ronca-Testoni et al., 1984; (4) The present study; (5) Yeung and Green, 1984.

the particulate fraction (Table 7). In a recent report of [^{3}H]NECA binding to guinea-pig lung membranes, Ukena and Schirren (1985) have demonstrated results similar to those observed in rabbit intestinal membranes. The K_D was 0.25 μM, the K_i values for NECA and 2-chloroadenosine were similar and R-PIA was more than 1000-fold less potent than NECA at [^{3}H]NECA binding sites. Furthermore, 8-phenyltheophylline did not displace the binding, although it competitively antagonized NECA-induced accumulation of cAMP in the lung slices.

Adenosine receptor agonists exhibit higher affinities at higher temperatures (Murphy and Snyder, 1982; Lohse et al., 1984). Although assay temperature of 0-4°C may explain in part the lower affinity of [^{3}H]NECA binding sites in these systems compared to that in striatum (37°C), this factor does not account for the anomalous specificity of the binding (Table 10).

The lack of effect of nucleoside transport inhibitors such as dipyridamole and NBMPR suggests that [^{3}H]NECA does not bind to the transporter sites. Ca^{2+}-channel antagonists such as nitrendipine and verapamil have been shown to affect radioligand binding to adenosine receptors in brain (Murphy and Snyder, 1983). However, neither compound was effective at [^{3}H]NECA binding sites in the intestinal membranes (Table 6). Although ATP displaced the binding of [^{3}H]NECA, its effect was evidently mediated through its breakdown product, adenosine, as it was not seen in the presence of adenosine deaminase (Fig. 30).

The Hill coefficient for most compounds were close to unity

except for theophylline and adenine, which were significantly lower than one. This may suggest that [^{3}H]NECA binds to two different sites in the membranes that are differentiated by theophylline and adenine only. The apparently linear Scatchard plot of the [^{3}H]NECA binding data may be due to a less than 10-fold difference in the affinity of [^{3}H]NECA at the two possible sites. Alternatively, the density of one of the sites may be much lower relative to the total number of binding sites.

The absence of effect of GTP or Gpp(NH)p (Table 7) indicate that the [^{3}H]NECA binding sites are not coupled to adenylate cyclase. However, as [^{3}H]NECA binding sites do not appear to be the physiologically relevant sites through which NECA produces smooth muscle relaxation, one would not expect to see an influence of guanine nucleotides on the binding. Thus, no conclusions can be drawn from these data, on whether or not the receptor couples with the enzyme adenylate cyclase in the intestinal smooth muscle.

It is, therefore, not possible to do more than speculate on the nature of the binding sites for [^{3}H]NECA in the intestinal membranes that this study has demonstrated. A large number of reports on [^{3}H]adenosine binding sites in various membrane preparations also describe properties inconsistent with those expected for adenosine receptors (Table 3). Daly et al. (1981) reported that binding of radioactive NCPCA (a 5'-derivative of adenosine with structure and properties similar to that of NECA) to brain membranes was displaced by adenosine derivatives and theophylline in a manner that differs from pharmacological findings. Furthermore, the binding of

[^{3}H]2',5'-dideoxyadenosine to putative P sites in rat brain membranes exhibits properties uncharacteristic of binding to the P site. For example, MTA has high potency at [^{3}H]2',5':dideoxyadenosine binding sites, although it has virtually no effect on the activity of adenylate cyclase (Nimit et al., 1982).

In summary, our attempts to label receptors that mediate relaxation of isolated intestinal muscle by adenosine were futile. Neither the widely used radioligands such as [^{3}H]R-PIA and [^{3}H]NECA, nor the newly developed [^{125}I]labelled R-IHPIA, proved useful. Radiolabelling of adenosine receptors in intestinal membranes, as well as in other systems, such as liver, platelets, thymocytes and lungs must, therefore, await development of radioligands that are characterized by high affinity, slow dissociation kinetics, and exclusive receptor binding, as is the case with [^{3}H]R-PIA, [^{3}H]CHA, and [^{125}I]R-IHPIA; the radioligands for brain and fat cell adenosine receptors. Linden et al. (1984) developed a new ^{125}I-labelled ligand N^6-iodobenzyladenosine ([^{125}I]IABA) and Stiles et al. (1985) have reported the use of [^{125}I]-labelled N^6-2-(4-aminophenyl)ethyladenosine ([^{125}I]IAPNEA) as a radioligand for adenosine receptors. [^{126}I]IABA exhibits much lower nonspecific binding in myocardial and brain membranes compared with [^{125}I]R-IHPIA. The utility of these radioligands as a probes for adenosine receptors in the intestine and other peripheral systems remains to be evaluated.

4.4 Delineation of the receptor subtype in the intestinal muscle

It has already been stated that adenosine receptors in rabbit

intestinal smooth muscle seem atypical. Although the rank order of potency for the relaxant action of various agonists conforms to that seen at A_1 receptors such as in brain (Baer and Vriend, 1985), neither inhibition nor stimulation of adenylate cyclase activity of these agents is demonstrable in the intestinal muscle membranes (Muller, [illegible]5). However, that components of the adenylate cyclase system such as the N protein (s) and the catalytic unit (c) are indeed functional in these membranes is indicated by the ability of GTP and of forskolin to stimulate the adenylate cyclase activity (Fig. 14). Furthermore, if the smooth-muscle relaxant action of adenosine and its derivatives was mediated through A_1 receptors (adenylate cyclase inhibitory), this would contradict the basic hypothesis of the involvement of cAMP in smooth-muscle relaxation (see sec. 1.3.7.3).

Although it is interesting and experimentally convenient that some A_1 receptors inhibit adenylate cyclase, while some A_2 receptors stimulate it, it is unlikely that all the effects of adenosine at cell membrane receptors are mediated through interaction with the adenylate cyclase system (Fredholm, 1982). It is now becoming increasingly clear that there are biological actions of adenosine that may not be related to cAMP. For example, although the adenosine receptors regulating transmitter release can apparently be classified as the A_1 subtype, and depression of cAMP content could conceivably cause a decrease in transmitter release, a direct role for cAMP in adrenergic neurotransmission has been questioned (e.g. Cubeddu et al., 1975; Clanachan, 1978; Fredholm, 1982). Similarly, the inhibitory effect of adenosine on neurotransmission in hippocampal slices is unlikely to be

mediated via cAMP (Dunwiddie and Fredholm, 1985). In both of these cases, interference with inward transport of Ca^{2+} would adequately explain the effects.

Modulation of Ca^{2+} fluxes in smooth muscles by adenosine has also been reported (see Sec. 1.3.7, 5.2). Furthermore, adenosine-induced smooth-muscle relaxation has also been linked to phosphatidylinositol (PI)-metabolism. A decrease in the phosphorylation of PI by adenosine has been reported in a subcellular fraction containing membranes and contractile proteins prepared from smooth muscle of calf aorta (Doctrow and Lowenstein, 1985). Besides adenosine, 5'-deoxy-5'-chloroadenosine (CDA), which has a vasodilatory action in isolated perfused rat heart (cf. Doctrow and Lowenstein, 1985), also decreases phosphorylation of PI in the aortic smooth muscle preparation. CDA-mediated inhibition of the enzyme phosphatidylinositol kinase in a smooth-muscle preparation from calf aorta is competitive for ATP, suggesting that the decrease in the phosphorylation of PI is due to a direct interaction of the nucleosides with the kinase. This effect of adenosine and CDA must, therefore, be mediated intracellularly. Various agonists at other receptors such as the alpha$_1$-adrenergic receptor, are known to affect the contractility of vascular smooth muscle by increasing the phosphorylation of PI (Lapetina et al., 1976; Villalobos-Molina et al., 1982; Villalobos-Molina and Garcia-Sainz, 1983; Takhar and Kirk, 1981). There is thus a correlation between contraction and metabolism of phosphatidylinositol in this tissue.

CDA produced a dose-dependent relaxtion of isolated small intestine from rabbit (Fig. 31) and the relaxant action was

competitively antagonized by SPT. Given the effect of adenosine and CDA on PI kinase in the aortic muscle, it will be of interest to determine if adenosine, acting via the extracellular receptors in the intestinal muscle, can use PI metabolism as the effector system. PI metabolism has also been implicated in contraction of guinea-pig intestinal muscle induced by substance P (Holzer and Lippe, 1985).

Furthermore, CDA (100 μM) also increased the activity of adenylate cyclase in murine neuroblastoma cell membranes (Fig. 12). Various adenosine derivatives stimulate the activity of neuroblastoma adenylate cyclase with an order of potency (NECA>R-PIA>S-PIA) which is consonant with the presence of A_2 receptors (Blume and Foster, 1980; Baer, unpublished data).

In the absence of demonstrable stimulation of adenylate cyclase in intestinal muscle by adenosine agonists, it is possible that the adenosine receptor in smooth muscle is different from the classical A_2 receptor such as in neuroblastoma cells. Historically, the classification of receptors has been based on the structure-activity relationships of a series of agonists and antagonists (Furchgott, 1972). Various adenosine derivatives that have a relaxant effect on isolated intestinal muscle (Table 8) also stimulate neuroblastoma adenylate cyclase, but the rank order of potency in the two systems is different. Thus, based on the agonists' order of potency, adenosine receptors in the two systems appear different. This is corroborated by our data obtained using a nucleoside antagonist of adenosine receptors.

Bruns (1980) showed that certain 5'-derivatives of adenosine such

as 5'-deoxy-5'methythioadenosine (MTA), are competitive antagonists of adenylate cyclase activity in VA13 fibroblast cell membranes (an A_2 receptor system stimulated by adenosine). We have demonstrated similar results using MTA in murine neuroblastoma cell membranes. In this system, NECA-induced stimulation of adenylate cyclase is competitively antagonized by MTA (Fig. 32). However, unlike the xanthine antagonist, SPT, MTA did not antagonize the agonist-induced relaxation of the isolated intestinal preparation. On the contrary, MTA caused relaxation of the isolated tissue and this effect was competitively antagonized by SPT (Fig. 31). MTA is a weak, but full, agonist in the intestine. Thus, by using this relatively less studied nucleoside antagonist, it has been possible to differentiate the adenosine receptor in intestinal smooth muscle from the classical A_2 receptor.

In conclusion, our efforts to characterize the adenosine receptor of intestinal smooth-muscle by means of radioligand binding studies were not successful. However, through the use of MTA, strong evidence has been adduced to show that the intestinal muscle receptor is different from the classical adenosine A_2 receptor. It is, therefore, possible that the intestinal smooth-muscle receptor is not coupled to adenylate cyclase, although as yet there is no direct evidence for this conclusion.

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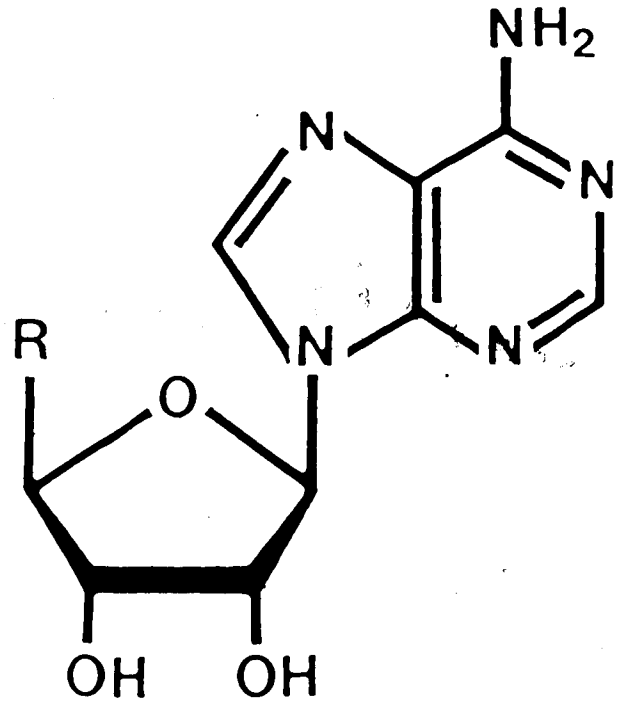
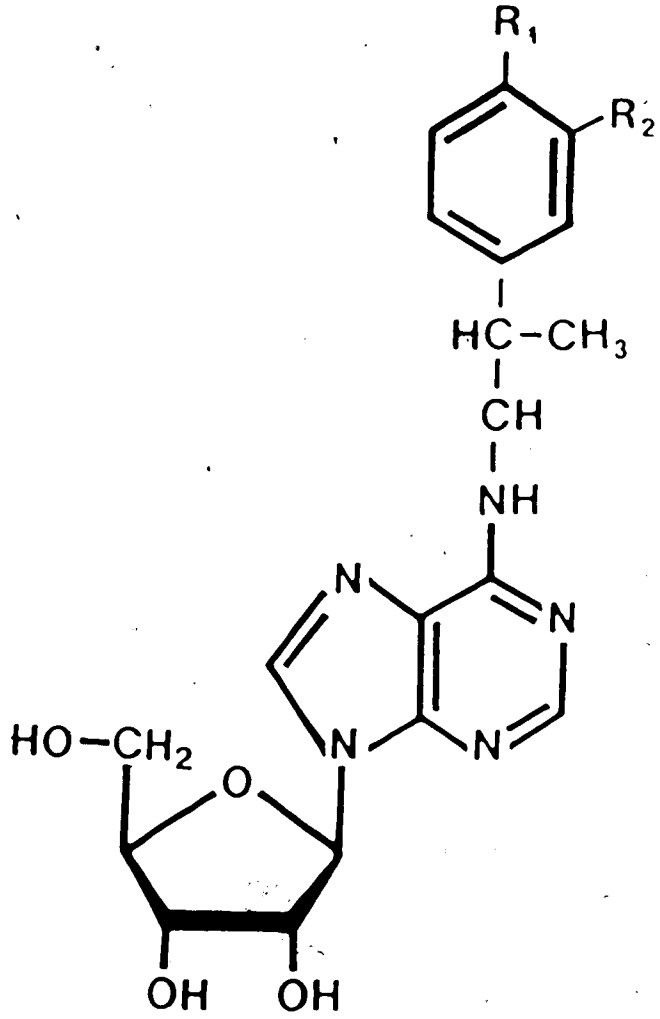
Appendix I - B. 5'-derivatives of adenosine



COMPOUNDS	R
Adenosine	- CH_2OH
5'-N-ethylcarboxamidoadenosine (NECA)	- $\overset{O}{\overset{\|}{C}}-HN-CH_2-CH_3$
5'-deoxy-5'-chloroadenosine (CDA)	- CH_2-Cl
5'-deoxy-5'-methylthioadenosine (MTA)	- CH_2-S-CH_3

Appendix I - A. N^6-derivatives of adenosine



COMPOUNDS

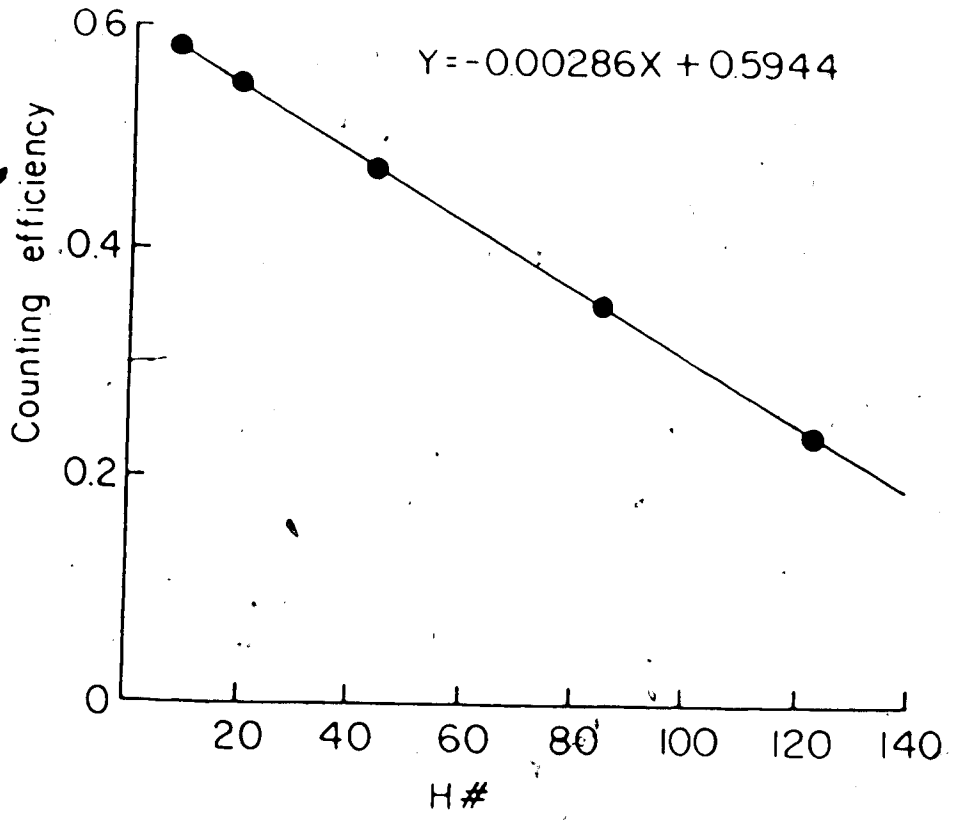
	R_1	R_2
N^6-(2-phenyl)isopropyladenosine (PIA)	H	H
N^6-[2-(4-hydroxy)phenylisopropyl]adenosine (HPIA)	OH	H
N^6-[2-(3-iodo,4-hydroxy)phenylisopropyl]adenosine (IHPIA)	OH	I

. . . Cont'd

Appendix II

The bases were supplied with their optical rotation designated. The assumption is made that p-OH substitution of amphetamine has no significance on the direction of optical rotation of the molecule and accordingly the (+)/(-) enantiomers correspond to the S/R-configurations, respectively, as is the case with amphetamine enantiomers and the derived diastereomers of PIA.

Appendix III



Quench Correction Curve