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Studies On Adenosine-mediated Neuromodulation In The Enteric Nervous System

Fedias Leontiou Christofi

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STUDIES ON ADENOSINE-MEDIATED NEUROMODULATION IN THE ENTERIC NERVOUS SYSTEM

by

Fedias Leontiou CHRISTOFI

Department of

Pharmacology & Toxicology

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ABSTRACT

Although the role of adenosine in the inhibition of acetylcholine release from myenteric neurons of the small intestine is firmly established, the classification and characteristics of such receptors have not been adequately described. Unlike in the central nervous system, it is not clear if heterogeneity of adenosine receptors exists, or if nucleoside-mediated inhibition of the release of other neuromediators occurs in the enteric nervous system. The studies reported in this thesis were directed at these questions.

Adenosine recognition sites were characterized in ligand binding studies at purified myenteric varicosities. The receptor nature of the binding sites labelled by selective adenosine analogs is supported by the saturability of binding, the good correlation between binding affinity and biological activity (ie. inhibition of Ach release) as well as the potency of adenosine receptor antagonists in displacing their specific binding.

Evidence was obtained for the presence of a heterogeneous population of adenosine receptors on enteric nerve endings. The data is consistent with the presence of A_1-like adenosine receptors with affinity for both N^6 - and 5'- substituted adenosine analogs, as well as A_2 adenosine receptors with affinity only for 5'-N-ethylcarboxamide adenosine. The A_2 receptor may be present in higher concentrations on enteric nerve endings.

Adenosine mediated inhibition of tachykinin release was demonstrated using cholecystokinin (CCK-8) and field-stimulated responses of atropinized guinea pig ileal strips. The data obtained indicates that the same adenosine receptor inhibits the release of both acetylcholine and tachykinins.

The presence of substantial quantities of alpha-neurokinin, substance P, met-enkephalin, leu-enkephalin and gastrin releasing polypeptide (GRP) in this synaptosomal preparation suggested its possible utility in functional studies on modulation of release of endogenous peptides by nucleosides. The finding of both potassium evoked- and GRP mediated-release of several neuropeptides from crude synaptosomal suspensions support this contention.

DEDICATION

TO MY MOTHER AND FATHER

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ABBREVIATIONS

Abbreviations are used extensively throughout this thesis. To assist the reader the following list of abbreviations should be of assistance.

NUCLEOSIDE ANALOGS

ADO, adenosine

CADO, 2-Chloroadenosine

CHA, N^6-cyclohexyl adenosine

CPA, N^6-cyclopentyl adenosine

MCPA, N^6-1-methylcyclopentyl adenosine

NCPCA, 5'-N-cyclopropylcarboxamide adenosine

R-PIA, N^6-[R-(-)-1-methyl-2-phenethyl]adenosine

R-PBA, N^6-[R-1-phenyl-2-butyl]adenosine

R-HBA, N^6-[R-1-hydroxy-2-butyl]adenosine

NECA, 5'-N-ethylcarboxamide-adenosine (systematic name: 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-ß-D-ribofuronamide)

2PAA, 2-phenylamino-adenosine

XANTHINE ANALOGS

THEO, theophylline

DPSPX, 1,3,-dipropyl-8-(4-sulfophenyl)xanthine

CPDPX, 8-cyclopentyl-1,3-dipropylxanthine

OTHER TERMS

LDH, Lactate dehydrogenase

PV, Partially purified myenteric varicosities.

P_2, Crude myenteric varicosity preparation

LMMP, longitutinal muscle-myenteric plexus strip

HPLC, high performance liquid chromatography

AA, atomic absorption

RIA, radioimmunoassay

V_m, membrane potential

NEUROPEPTIDES

SP, SPLI*, Substance P and Substance P - like immunoreactivity

alpha-NK, alpha-neurokinin

beta-NK, beta-neurokinin

ME, Met-enkephalin

LE, Leu-enkephalin

GRP, Gastrin Releasing Polypeptide

VIP, Vasoactive intestinal Peptide

GAL, Galanin

* LI at the end of each abbreviation refers to -like immunoreactivity

PYY, Neuropeptide PYY

SP-A, [d-pro^4,d-trp^{7,9,10}] (SP) (4-11)

TTX, tetrototoxin

INTRODUCTION

The actions of adenosine and related compounds on nerve function suggest that this nucleoside plays a physiological modulatory role in neurotransmission. Evidence for modulation of release of several transmitters in both the central and peripheral nervous systems by adenosine has been reported, and these findings have been the subject of many reviews (Fredholm and Hedqvist, 1980; Phillis and Wu, 1981; Stone, 1981; Ribeiro, 1981; Daly et al, 1983; Stone, 1985; Burnstock, 1981; Snyder, 1985).

The use of stable, nonmetabolizable analogs of adenosine such as R-PIA, NECA, CADO, S-PIA and CHA in preparations of central neural origin has permitted the designation of adenosine receptor subtypes as A_1 and A_2 (van Calker et al, 1979; Schwabe, 1983). These two classes of extracellularly located adenosine receptors were defined, on the basis of the ability of nucleosides to inhibit (A_1 or R_i) or stimulate (A_2 or R_a) the activity of adenylate cyclase. Relatively high affinity A_1 adenosine receptors (Londos et al, 1980) mediate inhibition of adenylate cyclase, while lower affinity A_2 receptors mediate stimulation of adenylate cyclase (Daly et al, 1981; Bruns, 1980). The prototypical adenosine receptor antagonists theophylline and caffeine are non-selective for A_1 and A_2 receptors (Daly, 1982; Fredholm & Persson, 1982; Daly et al, 1983). According to this classification, adenosine itself has activity at nanomolar concentrations for A_1 receptors but activity at micromolar concentrations at A_2 receptors.

Subclasses of stimulatory A_2 receptors linked to adenylate cyclase also exist in the central nervous system, and an attempt has been made to catagorize these stimulatory A_2 adenosine receptors into high (EC_{50}, 0.5 uM) and low affinity (EC_{50}, 10-20 uM) subclasses (Premont et al, 1979; Londos et al, 1983; Daly et al, 1983). The high affinity A_2 receptors appear to be localized on neurons of the striatum (Premont et al, 1983; Wojcik et al, 1983). Neurochemical studies have indicated that the 5'-N-substituted carboxamides of adenosine (eg. NECA) are about an order of magnitude less potent at the A_1 receptor than R-PIA and CHA and 5 to 50 times more potent at the A_2 receptor (Daly, 1982; Londos et al, 1983).

Since the involvement of adenylate cyclase in the responses to nucleosides was not always clear, the criteria for classification of adenosine receptor subtypes were revised (Stone, 1984), with the emphasis being on the potency differential between A_1 and A_2 receptor agonists. Those tissues in which the potency of R-PIA and CHA > NECA are described as possessing A_1 receptors and those tissues in which this potency order is reversed display A_2 receptor characteristics. Although the A_1 receptor was designated as having a marked stereospecificity for the diastereoisomers of PIA (ie. the R(-)-isomer is 50 to 100 fold more potent than the S(+)-isomer) in contrast to the A_2 receptor, some controversy exists regarding the delineation of A_1 and A_2 receptor subtypes on the basis of their responses to the diastereoisomers of PIA (Smellie et al, 1979; Fredholm et al, 1982; Edvinsson and Fredholm, 1983).

Ribeiro and Sebastiao (1986) have recently proposed yet another adenosine receptor subtype to describe the receptor present on excitable tissues ie. in nerve endings and heart. This A_3 adenosine receptor is not coupled to adenylate cyclase and has the agnonist profile R-PIA, CHA, NECA > CADO, where S-PIA is usually less potent than CADO. This proposal is in keeping with previous suggestions (Phillis & Wu, 1981; Stone, 1985) about the need for a third adenosine receptor mediating the electrophysiological effects of adenosine on the central nervous system. The A_3 adenosine receptor is believed to be linked to calcium which is needed for transmitter release. There is speculation that this receptor may represent the voltage-dependent calcium channel.

In the peripheral nervous system, nucleosides have been shown to inhibit acetylcholine (Ach) release from myenteric nerves (Vizi & Knoll, 1976; Sawynok & Jhamondas, 1976; Hayashi et al, 1978) of the small intestine, by interacting with prejunctional adenosine receptors located on varicose nerve terminals (Sawynok & Jhamandas, 1976; Gustaffson et al, 1978; Okwuasaba et al, 1978; Cook et al, 1979; Paul et al, 1982). Adenosine has been shown to inhibit Ach release by suppressing the presynaptic influx of $[Ca^{++}]$ (Shinozuka et al, 1985). These findings suggest that endogenously released adenosine may have an important neuromodulatory role in the peripheral nervous system.

Despite the considerable stucture activity data availabe at this site

(Maguire and Satchell, 1979; Hayashi et al, 1977; Paul et al, 1982), an attempt to classify the peripheral adenosine receptor into A_1 and A_2 subtypes using the stimulated guinea pig ileum preparation, has met with limited success (Paton, 1981). Paton (1981) defined the presynaptic receptors in peripheral cholinergic nerve terminals as A_1, even though the 5'-N-carboxamide NCPCA was clearly more potent than R-PIA. This conclusion was based on the stereospecificity of PIA, and the author (Paton) could have justifiably also concluded, that an A_2 adenosine receptor was involved on the basis of the higher potency displayed by NCPCA. As no other studies attempted to resolve this issue, it was clear that a more rigorous approach to the characterization of the peripheral adenosine receptor(s) was necessary.

Ligand binding studies carried out on broken-cell preparations of brain tissue have permitted the fundamental characteristics of affinity and density of binding sites to be determined for several nucleoside ligands. Successful identification of A_1 adenosine receptors in the CNS was achieved using the agonist radioligands [^{3}H]-CHA (Bruns et al, 1980), [^{3}H]-R-PIA (Schwabe & Trost, 1980) and [^{3}H]-CADO (Williams & Risley, 1980; Wu et al, 1980). In contrast, at the peripheral nervous system comparable information was lacking. Despite the extensive structure-activity information available, the fundemental characteristics of the adenosine receptor on enteric nerve endings were not known.

The utility of the isolated myenteric nerve ending preparation in the investigation of nucleoside regulation of transmitter release has been well documented (White & Leslie, 1982; Reese and Cooper, 1982). Thus, isolated myenteric varicosities (autonomic synaptosomes) (PV) prepared from the guinea pig ileum were used as the substrate in ligand binding studies aimed at characterizing peripheral adenosine receptor(s) located on myenteric nerve endings. The choice of substrate was based on the premise that the PV contains the functional adenosine receptor(s) mediating the inhibition of transmitter release at the stimulated ileum. To test this possibility, the relative affinities of adenosine analogs as displacers of binding of [^{3}H]-NECA, [^{3}H]-CHA and [^{3}H]-R-PIA to PV were compared with their relative potencies as inhibitors of Ach release in the biological system (ie. the source of the PV). It was also necessary to classify the adenosine receptor(s) in the functional system according to the potency profile of several selective adenosine receptor agonists as inhibitors of Ach release at the stimulated ileum. The fundemental characteristics of adenosine recognition sites on PV were determined in saturation binding studies using [^{3}H]-CHA and [^{3}H]-NECA.

In the CNS, there is clear evidence for heterogeneity of adenosine receptors (ie. A_1 and A_2 subtypes) revealed in ligand binding studies using the mixed A_1/A_2 adenosine receptor ligand [^{3}H]-NECA (Yeung and Green,1983). In addition to its binding to A_1 and A_2 adenosine receptors, [^{3}H]-NECA may be involved in the binding to subtypes of the A_2 receptor (Bruns et al, 1986). Studies with [^{3}H]-NECA binding at

peripheral tissues were less conclusive in identifying sites which had characteristics of specific adenosine receptor sites (Schutz et al, 1982; Huttemann et al, 1984; Bruns et al,1986). Autoradiographic data obtained from the myenteric plexus of the guinea pig ileum (Buckley & Burnstock, 1983) suggested that NECA may label a multiplicity of binding sites which are not all recognized by the A_1 ligand CHA. Since no other information about the binding of these ligands to myenteric nerves was available, the biological relevance of these sites was uncertain.

The possible existence of multiple adenosine receptors in the periphery was thus investigated in binding studies which involved displacement of binding of the mixed A_1/A_2 ligand [^{3}H]-NECA or the A_1 selective ligand [^{3}H]-CHA from PV by selective adenosine receptor agonists and xanthine antagonists. The displacement profiles thus obtained could provide useful data, towards the delineation of the peripheral adenosine recognition sites into A_1, A_2 and/ or A_3 types, according to the current criteria.

The ability to characterize the interaction of antagonists with the adenosine receptor(s) present in the isolated ileal preparation and thus to obtain the affinities of the antagonists, would permit possible receptor heterogeneity to be revealed in a functional system (Kenakin, 1984). The non-selective adenosine receptor antagonist theophylline and the relatively A_1 selective antagonist DPSPX were used in Schild analysis in which N^6-substituted analogs (used as A_1 agonists) and 5'-

N-carboxamides (used as A_2 agonists) were the agonists. These studies would permit a useful comparison to be made between binding and functional data.

Although clear evidence exists for nucleoside inhibition of Ach release from myenteric nerves, the putative inhibitory effect of nucleosides on such release of other excitatory neuromediators is not clear. Radioimmunochemical and immunohistochemical studies have demonstrated that many peptides are present in neurons of the myenteric plexus and that they may function as transmitters or neuromodulators. Considerable evidence exists suggesting that substance P may function as an excitatory neurotransmitter in the enteric nervous system (Furness & Costa, 1981; Holzer,1983; Baron, 1984) and a preliminary report by Bartho (1985) provided indirect evidence, using adenosine receptor antagonists, that nucleosides may play a role in the regulation of substance P release from enteric nerves. However, the nature of the adenosine receptor(s) involved in this function has not been elucidated. Therefore, structure activity studies using selective adenosine receptor agonists together with Schild analysis were carried out, using the atropinized LMMP strip, in an attempt to identify the adenosine receptor subtype(s) involved in the inhibition of tachykinin release. Using this approach, the type of adenosine receptor(s) mediating inhibition of Ach release could be compared with the type(s) mediating inhibition of tachykinin release.

The issue of whether alpha-neurokinin is also released from myenteric

nerves and is subject to nucleoside regulation was also unresolved. Parallel studies using the isolated myenteric varicosity preparation were required to elucidate, both the identity of the tachykinin(s) released as well as their possible regulation by nucleosides. As this in vitro preparation had not been used in the past to investigate the release of endogenous neuromediators from enteric nerves, it was necessary to firmly establish both the feasibility and suitability of this model system for such studies. Both the basal and depolarization-induced release of TKLI as well as other peptide-immunoreactivities were therefore characterized.

On the basis of this introduction the following questions were addressed in the work that was the foundation for the thesis:

(1) What are the characteristics of adenosine receptors on myenteric plexus synaptosomes ?

(2) Does heterogeneity of adenosine receptors exist on enteric nerve endings ?

(3) Does adenosine inhibit the release of endogenous substances from these nerves ?

(4) Is the isolated myenteric nerve ending preparation a suitable model system for the study of modulation of endogenous neuropeptide release by nucleosides ?

HISTORICAL REVIEW

This brief historical review will present selected background material relating to nucleosides and related purines as well as the mammalian tachykinins. In particular, attention will be focussed on the effects of these substances on neurotransmission in the myenteric nervous system of the small intestine.

Historical background.

Nucleosides/Nucleotides

At the beginning of the century it was suggested by Elliott (1905) and Langley (1905) that active substances in tissue fluids and extracts stimulate excitable tissues by interacting with specific 'receptive substances' or receptors. Further support for this contention came from studies which showed that both the transmission and the action of applied substances could be specifically blocked, as originally demonstrated for the action of ergot (Dale, 1909). In the parasympathetic division of the autonomic nervous system, it was suggested that nerve activation released a muscarine-like substance (Dixon, 1906) and the effects of nerve stimulation could be mimicked by muscarine and by choline esters, especially acetylcholine (Dale & Ewins, 1914; Dale, 1914). Later, it was shown by Loewi that in the frog heart, vagal stimulation resulted from a chemical agent much like acetylcholine (Loewi, 1921; Loewi & Navratil, 1926). With the isolation of Ach in mammalian tissues (Dale & Dudley, 1929), the concept of chemical neurotransmission became widely accepted. That

same year, Drury and Szent-Gyorgi (1929) documented effects of adenosine on the cardiovascular system but its short half life confounded attempts to use adenosine as an antihypertensive agent (Honey et al, 1930). More than two decades later, Feldberg & Sherwood, (1954) documented the depressant effects of adenosine on the CNS. Despite such early observations, examination of the responses mediated by adenosine at a molecular level has only taken place in the last 20 years with the discovery of the second messenger cAMP and the pioneering work of Sattin & Rall (1970) who demonstrated the effect of adenosine in the modulation of cAMP levels. Burnstock et al (1970) proposed the purinergic nerve hypothesis postulating ATP as the neurotransmitter of the non-cholinergic, non-adrenergic nerves of the guinea pig ileum. This hypothesis has been a source of inspiration for many subsequent studies. In 1971, Ginsborg & Hirst discovered that adenosine could reduce the output of transmitter from the rat phrenic nerve endings. The role of adenosine and related purines in purinergic neurotransmission in both the central and peripheral nervous systems has been the subject of many reviews (Burnstock, 1972, 1979; Ribeiro, 1978; Fredholm and Hedqvist, 1980; Phillis & Wu, 1981; Stone, 1981; Daly et al, 1981; Stone, 1981; Su, 1983; Williams, 1983; Snyder, 1985, Williams, 1987).

Tachykinins.

Among the many endogenous substances which have been identified, neuropeptides are the newest class of molecules which may function

as neurotransmitters or neuromodulators in both central and peripheral nervous systems. Probably the best characterized of these is substance P which was detected in extracts from various tissues, by von Euler & Gaddum in 1931. His early observations that large amounts of SP exist in the intestinal wall, together with its powerful spasmogenic effects, suggested that this peptide may be involved in the control of intestinal motility (von Euler, 1936). Characterization of its pharmacological effects as well as its distribution was carried out in the following three decades. However, lack of chemically pure preparations and specific analytical methods hampered progress in the field. While attempting to isolate a corticotropin-releasing factor from the bovine hypothalamus, Leeman & Hammerschlag (1967) accidentally discovered a peptide sialogogue which was not blocked by cholinergic or adrenergic antagonists. Its isolation and chemical characterization in 1970 (Chang & Leeman, 1970, 1971) and later synthesis (Tregear et al, 1971) made it possible to develop specific antisera for SP which would allow detailed study of its distribution and release. For a detailed reviews on substance P, see Pernow (1983), Jordan & Oehme (1985) and Bartho & Holzer (1985).

Recently it has become apparent that substance P represents only one member of a structurally related family of bioactive peptides called tachykinins. Several tachykinins other than substance P were isolated from non-mammalian tissues and these included eledoisin, physalaemin and phyllomedusin (Erspamer & Anastasi,1962; Erspamer et

al,1964; Anastasi & Erspamer,1970). Because of their fast onset of action on tissues of the gut as compared to the slower acting bradykinins, the term tachykinin was given to this group. Most of their biological activity was later found to depend on the conserved carboxyl-terminal sequence (-Phe-X-Gly-Leu-Met-NH_2).

From extracts of mammalian spinal cord, a peptide named substance K (so named to reflect its structural homology with the amphibian tachykinin kassinin) was discovered (Maggio et al,1983) and was first sequenced by Kimura et al (1983). These authors reported the sequence of a novel tachykinin isolated from extracts of porcine spinal cord, which they named alpha-neurokinin, which later proved to be identical to bovine substance K. They also identified another tachykinin named beta-neurokinin. At the same time Kangawa et al (1983) isolated and sequenced a peptide they called neuromedin K, which proved to be identical to beta-neurokinin, and neuromedin L, which proved to be identical to alpha-neurokinin or substance K.

Classification of adenosine receptors.

Burnstock (1978) proposed the existence of two types of cell surface purinergic receptors, on the basis of pharmacological studies carried out using a variety of peripheral tissues. The P_1 receptor is coupled to adenylate cyclase, responds to adenosine and is blocked by the methylxanthines. The P_2 receptor is sensitive to the nuclectide ATP, has no definitive antagonist and its activation may

be related to increased prostaglandin synthesis. The light sensitive ATP antagonist arylazidoaminoproprionyl ATP ($ANAPP_3$) (Hogaboom et al, 1980) seems to be more effective against excitatory than inhibitory actions of ATP (Frew and Lundy, 1982).

At about the same time, other investigators (Londos & Wolff, 1977; Londos et al, 1980) identified two subclasses of the P-1 purinergic receptor by studying the effects of nucleoside analogs on cAMP production in several different cultured cell lines. Adenosine agonists in which the ribose moiety was unchanged were shown to potently inhibit cAMP production, in the nanomolar range, in Leydig tumor cells, liver cell preparations and adipocytes by interacting with specific receptors (Londos et al, 1980). At micromolar concentrations, adenosine agonists with an intact ribose moiety, were shown to stimulate cAMP production. The receptor subtypes mediating inhibition or activation of adenylate cyclase were designated as R_i or R_a respectively. Van Calker et al (1978, 1979) obtained similar results from cell lines of glial character, but they subclassified the receptors as A_1 and A_2 types. The A_1 and A_2 nomenclature is more commonly used than the R_i/R_a nomenclature.

An intracellular nucleoside recognition site termed the P-site has also been described but its physiological significance is unclear. Its activation by dideoxyadenosine causes inhibition of adenylate cyclase (AC), but unlike either A_1 or A_2 receptors, this site does not appear to be antagonized by methylxanthines (eg. theophylline),

but rather by 5'-methylthioadenosine. This site has different structural requirements for agonists from either A_1 and A_2 receptors (Londos et al, 1979) and has not been extensively studied by ligand binding studies. This site is clearly not a receptor site in the normally accepted sense and an endogenous ligand has not been identified.

A classification of adenosine receptors which is based on rank order of potencies of selective adenosine analogs has advantages over the relationship to adenylate cyclase activity since evidence exists for coupling of adenosine receptors to second messenger systems other than adenylate cyclase. Adenosine receptors in the heart were shown to be coupled to cardiac K^+ conductance via the guanine nucleotide regulatory protein and the adenosine-mediated changes in K^+ conductance occur without changes in cAMP levels (Bohm et al, 1986).

A comprehensive review by Ribiero & Sebastio (1986) discusses the evidence suggesting that nucleosides inhibit transmitter release by a mechanism which may be independent of its ability to modify cyclic AMP levels or adenylate cyclase activity. Briefly, these investigators suggested that an A_3 adenosine receptor exists on excitable tissues, which mediates inhibitory effects of adenosine on transmitter release. Although the mechanism of inhibition is not known, it is clear that calcium is involved in mediating the inhibitory responses to adenosine.

Effects of adenosine on Acetylcholine release from myenteric nerves of the small intestine.

One of the best characterized biological actions of adenosine is inhibition of the release of neurotransmitters, an action that has been clearly established at the neuromuscular junction (Ginsborg and Hirst,1972) as well as at synapses in the sympathetic nervous system (Fredholm ,1976; Clanachan et al,1977). At these sites aderosine appears to have few, if any, postsynaptic actions. Electrophysiological measurements of the quantal release of transmitter (eg.Silinsky, 1984), have confirmed that the primary action of adenosine at such synapses is a reduction of the number of quanta released by a nerve stimulus.

Inhibition of acetylcholine (Ach) release from the myenteric nervous system of the small intestine by nucleosides has been well characterized in recent years. Adenosine was shown to inhibit contractile responses to transmural nerve stimulation of the guinea pig small intestine, without altering the responsiveness of the ileum longitutinal muscle to stimulation with histamine (Gustaffson, 1978), Ach (Sawynok & Jhamandas, 1976; Vizi & Knoll, 1976; Hayashi et al, 1978; Gustaffson et al, 1978) or electrical stimulation with pulses of long duration (Gustaffson et al, 1978). These data provided indirect evidence for a prejunctional site of action of adenosine on the release of Ach from the small intestine.

The inhibitory effect of adenosine on transmural stimulation was

shown to be frequency dependent and rapidly reversible (Gustaffson, 1978). At low frequencies adenosine inhibition of contractile responses was observed at adenosine concentrations slightly higher than 100nM with maximum inhibition occuring at 0.1mM. At higher frequencies of stimulation the effect of adenosine was weak and complete inhibition was difficult to obtain. Similar conclusions were drawn from studies measuring the release of [3H]-Ach (Hayashi et al, 1978), bioassay of Ach release (Vizi & Knoll, 1976; Hayashi et al, 1978) and direct measurement of Ach release by gas chromatography-mass spectrometry (Gustaffson et al, 1980).

The release of nucleosides from the small intestine during nerve stimulation has been demonstrated by many investigators (Burnstock et al, 1970; Su et al, 1971; Satchell & Burnstock, 1971). This, together with demonstration of depolarization-induced release of ATP from isolated myenteric nerve endings of the small intestine (White & Leslie,1982), is consistent with a neurotransmitter and / or neuromodulator role for adenosine or a related purine in the myenteric nervous system. As judged from K^+-induced neurogenic responses (Gustaffson, 1978), adenosine appears to act directly on the nerve terminals.

Whether the endogenous levels of adenosine released from myenteric neives were sufficient to cause the inhibition observed with exogenous application of adenosine, was investigated by quantitating the adenosine levels released from guinea pig ileal preparations in

the surrounding bathing fluid. During rest, adenosine was present at concentrations slightly below those necessary for modulation of the neurotransmission by exogenous adenosine. Application of agents which inhibited metabolism of adenosine or transmural nerve stimulation caused an increase in adenosine levels towards or into the inhibitory concentration range (Gustaffson, 1980).

Cyclic AMP and 5'-AMP, as well as ATP, have been suggested as potential sources of extracellular adenosine (Newman and Mcllwain, 1977; Pons et al, 1980; MacDonald & White, 1984) and the release of any of these adenine nucleotides, either on their own (Potter and White, 1980) or as co-transmitters (Israel et al, 1980; Burnstock, 1983) results in their rapid degradation by synaptic ectoenzyme systems to the nucleoside which can interact with specific cell surface receptors to modulate transmitter release.

Gustaffson et al (1980) demonstrated that theophylline competitively antagonized the actions of adenosine on contractile responses elicited by transmural nerve stimulation but had little or no effect on cAMP levels measured in homogenates of similar preparations. In contrast, three other inhibitors which are somewhat selective for phosphodiesterase were found to enhance the inhibitory actions of adenosine (Gustaffson et al, 1980). These findings suggested to the authors that cAMP formation may be involved in the action of adenosine.

Ligand binding studies at the myenteric nervous nervous system.

Autoradiographic localization of [^{3}H]-NECA binding sites (Buckley & Burnstock, 1983) revealed a heterogeneous labelling of guinea-pig intestine with heavy labeling over the enteric ganglia and in clusters over the mucosa; a low level of label was homogeneously distributed over the muscularis externa. No binding sites were evident using [^{3}H]-CHA although both [^{3}H]-CHA and [^{3}H]-NECA binding sites were localized over comparable areas of rat brain. CHA attenuated the specific binding of [^{3}H]-NECA by only 35% which suggested that NECA reveals a multiplicity of binding sites, only partially available to CHA. The inability of labeled CHA to reveal any specific binding sites on intestinal tissue suggested to the authors, either a low number of binding sites or a low affinity of the ligand for the binding sites exists. It was suggested that the specific labeling seen over the muscularis externa and myenteric ganglia may be related to both postjunctional and pre-synaptic actions of NECA on guinea pig small intestine. The only other ligand binding study on small intestinal tissue was reported by Williams & Velantine (1985), who showed that [^{3}H]-CHA binds to guinea pig ileal synaptosomal membranes with high affinity, which is consistent with the presence of an A_1 adenosine receptor at this locus, similar to that found in CNS membranes.

Adenosine receptor subtypes on excitable and non-excitable tissues.

The existence of A_2 adenosine receptors has been demonstrated in non-excitable tissues such as human lung mast cells (Hughes et al, 1984), platelets (Ukena et al, 1984), renal cortex (Churchill and Churchill, 1985),turkey erythrocytes (Braun & Levitzki, 1979) and human neutrophils (Cronstein et al, 1985). As demonstrated in peripheral tissues, [^{3}H]-NECA binds to human neutrophils and human platelets at A_2 receptors with K_d's of .22uM and .16uM respectively. Selective adenosine agonists inhibited binding of [^{3}H]-NECA to human neutrophils with a rank order of NECA = 2CADO > R-PIA. Such binding to platelets was inhibited with a potency profile of NECA > 2CADO > ADO but CHA and R-PIA did not significantly affect [^{3}H]-NECA binding, an observation not consistent with the stimulation of adenylate cyclase at this tissue. Such findings demonstrate the differences between [^{3}H]-NECA binding sites in the periphery and the brain.

Rat fat cells were reported to possess A_1, A_2 and P receptors (Garcia-Sainz & Torner,1985). These receptors were revealed by the selective action of pertussis toxin. That is, A_1 receptor-mediated actions are abolished, A_2-mediated actions are revealed and P-mediated actions are not affected. In guinea pig trachealis muscle, evidence was presented for the existence of A_1 and A_2 adenosine receptors on the basis of potency orders of several selective agonists for adenosine receptors. The relaxation responses followed

a potency order of NECA > R-PIA > CPA > CHA consistent with an A_2 interaction. In contrast, the contractile responses in naive trachea followed an analog potency order indicative of an A_1 receptor subtype (R-PIA > 2-CADO = CPA = CHA and NECA only relaxed these tissues.

In excitable tissues, the A_2 adenosine receptor is preferentially located on the smooth muscle cells of the trachea (Brown and Collis, 1982), taenia coli (Burnstock et al, 1984) and blood vessels (Collis and Brown, 1983; Kusachi et al, 1983; Edvinsson and Fredholm, 1983), mediating relaxation of these tissues.

Most of the evidence suggests that nerve endings do not possess functional A_2 adenosine receptors. As exceptions, decrease in transmitter release in the rat portal vein (Kennedy & Burnstock, 1984), but not in the rabbit portal vein (Brown and Collis, 1983) appear to involve A_2 adenosine receptors. The decrease in the firing rate of cortical neurons (Phillis, 1982; Stone, 1982) has also been suggested to be mediated via A_2 adenosine receptors. However, as Dunwiddie et al (1984) pointed out, the observed potency order for analogs may have resulted from their local application in situ.

<u>Ligand binding studies and adenosine receptor heterogeneity.</u>

Ligand binding studies have demonstrated directly that high affinity

binding sites which may mediate responses to extracellular adenosine exist. The use of ligand binding techniques to characterize adenosine receptors, permits analysis of receptor subtypes and mechanisms of action of drugs (Snyder, 1984). Rapid metabolism by adenosine deaminase precluded the use of [^{3}H]-adenosine as a ligand. The methylxanthines, caffeine and theophylline were shown to have only micromolar potencies at adenosine receptors and therefore were not suitable as ligands. Several reports described binding sites for [^{3}H]-Adenosine, with dissociation constants in the micromolar range and equivocal pharmacology (Schwabe et al, 1979; Newman et al, 1980; Williams, 1981). Snell and Snell (1983) recently described high affinity binding sites in NG108CC15 cells and brain tissue. However, in general [^{3}H]adenosine may be considered a poor ligand for binding to adenosine receptors (Newman, 1983).

With the synthesis of stable adenosine analogs in 1980, ligand binding studies proved fruitful in characterizing adenosine receptors in brain tissue (Bruns et al, 1980; Schwabe and Trost, 1980). The diastereoisomers of N^6-(4-hydroxyphenyl)-isopropyladenosine (HPIA) which were recently synthesized, iodinated and used as radioligands for adenosine receptors in rat brain appear promising ligands for characterizing high affinity adenosine receptors (Munshi et al, 1985).

Some effort has also been made to identify P_2 receptors by ligand binding techniques. High affinity (K_d = 20 nM) [^{3}H]-ATP binding to

rabbit urinary bladder has been described (Levin et al, 1983), such binding being distinct from that involving divalent ion ATPase activity. Binding of the stable analog of ATP, AppNHp, had a dissociation constant in the nanomolar range, but its binding was irreversible. Considering the evidence that there might be distinct P_2 receptors in the CNS (Jahr and Jessell, 1983) and the peripheral nervous system (Burnstock, 1978), it is likely that more suitable ATP radioligand binding assays will soon be developed. It should be noted, that without appropriate physiological and pharmacological validation (Hrdina, 1985), binding sites described by specific radioligands cannot be described as receptors.

On the basis of their pharmacological properties, their ability to bind labeled adenosine analogs and their effects on AC, several adenosine receptor subtypes have been distinguished in the CNS. The A_1 adenosine receptor has been shown to have the highest affinity for adenosine analogs of any adenosine binding site, such that even A_2-selective ligands (eg. NECA) have a higher affinity for A_1 than A_2 sites. The A_1 binding site has been studied with [^{3}H]-2CADO (Williams & Risley, 1980), R-phenylisopropyladenosine (Schwabe & Trost, 1980) and cyclohexyladenosine (Bruns et al, 1980; Murphy & Snyder, 1982). In a recent review, Williams (1987) suggested that there may be multiple A_1 receptor sites, but this is still uncertain. The binding sites that correspond to the A_2 receptor bind adenosine analogs with a lower affinity than does the A_1 site.

The adenosine analogue [^{3}H]-NECA has a relatively high affinity for the A_2 site and has been used in radioligand binding studies to characterize such sites (Yeung & Green, 1984). Both Hutteman et al (1984) and Yeung & Green (1984) showed that labeled NECA binds both to A_1 and A_2 receptors. A_2 receptor binding can be studied by treating membranes with N-ethylmaleimide, which degrades A_1 but not A_2 receptors, or by including 50nM CPA in the incubation to selectively block A_1 receptors (Bruns et al, 1986).

Adenosine analogs can increase cAMP levels in many areas of the brain which do not have high affinity A_2 binding sites (Dunwiddie, 1985). This has led to the suggestion that subtypes of the A_2 receptors are selectively localized in some brain regions, while the lower affinity A_2 site is more widely distributed.

The use of nonmetabolizable analogs of ATP, including the alpha, beta- and beta, gamma-methylene isosteres, have provided definitive evidence for unique ATP recognition sites. Reliable antagonists for the P_2 receptor have proved difficult to identify. 3-O-3[N-(4-Azido-2-nitrophenyl)amino]proprionyl-ATP ($ANAPP_3$) has been a useful tool. It has been shown to be a photolabile irreversible blocker of P_2 receptors (Fedan et al, 1985). The stable ATP analog, [^{3}H]AppNHp, bound irreversibly to rat brain membranes with affinities of 1nM (Williams & Risley, 1980).

Ligand binding studies have revealed receptor heterogeneity in many areas of the brain. Murphy and Snyder (1982) investigated A_1 receptor sites using the radioligands R-PIA, CHA and DPX. Species differences were observed in terms of ligand affinity, stereospecificity of PIA diastereoisomers as well as the number of binding sites labeled. Recent studies with the potent xanthine antagonist PAPCX (Bruns et al, 1983) have revealed the presence of distinct differences in binding characteristics of A_1 receptors between brain and the heart (Burnstock & Hoyle, 1985). N-ethylamaleimide or 5'-guanylylimidodiphosphate treatment, resulted in three affinity states for the A_1 receptor in brain tissue (Yeung & Green, 1983).

Characterization of adenosine receptors located on myenteric nerve endings of the small intestine.

The cell surface location of adenosine receptors on myenteric nerve endings was demonstrated using an analog of adenosine, covalently linked to a large molecular weight, impermeant sugar (Okwuasaba et al, 1978). Similar findings were obtained using this approach on dog coronary myocytes (Olsson, 1976).

Vizi & Knoll (1976) presented evidence showing that the inhibitory effect of purines on the evoked release of Ach from the myenteric plexus of the guinea pig ileum was similar in the presence of excess calcium (5mM) compared with normal calcium (2.5mM). However, this

preparation was not very sensitive to changes in calcium, since increase in the calcium concentration in the bathing fluid did not cause a significant increase in the evoked release of Ach.

Dowdle & Maske (1980) correlated the inhibition of the smooth muscle contraction, inhibition of Ach release from postganglionic neurons in the myenteric plexus, and calcium concentration in the bath. They found that calcium deprivation enhanced the inhibitory effect of adenosine and calcium excess antagonized the effect of adenosine. Low calcium concentrations in the bath and the adenosine uptake blocker dipyridamole acted synergistically to potentiate the inhibitory effect of adenosine on cholinergic transmission. The inhibitory potency of adenosine was decreased in the presence of the calcium ionophore A23187. In addition, calcium excess and theophylline synergistically reversed the inhibitory effect of adenosine.

Hayashi et al (1981), demonstrated that the log concentration - response curves for the inhibitory effect of adenosine on the twitch contractions in the guinea pig ileum is shifted to the left in low calcium (0.9mM) and to the right in high calcium (3.6mM). These investigators also showed that theophylline mimics the effects of high calcium on the dose-response curves. It was concluded from these studies that adenosine may inhibit Ach release by either interfering with the influx of calcium or the availability of calcium in cholinergic nerve terminals.

In a recent study correlating Ach release and calcium uptake in synaptosomes from the guinea pig ileum myenteric plexus, it was demonstrated the adenosine reduces the uptake of calcium in both potassium and electrically-stimulated synaptosomes (Shinozuka et al, 1985). Adenosine was shown to completely inhibit the ^{45}Ca uptake induced by electrical stimulation, whereas only a small inhibition of calcium uptake was observed in K^+-depolarized synaptosomes. The adenosine-induced inhibition of [^3H]-Ach release was also more pronounced in the electrically-stimulated than in the potassium-depolarized synaptosomes. Shinozuka et al (1985) suggested that either at high K^+-concentrations the influx of calcium is so intense as to mask the inhibitory action of adenosine or, the depolarization induced by high potassium might stimulate another mechanism of calcium entry, which is adenosine insensitive, in addition to the adenosine-sensitive calcium influx mechanism. These authors pointed out that the potassium depolarization-induced calcium influx and Ach release seems to be different from the electrically-induced excitation as well as from the physiological excitatory response. These studies clearly demonstrate the ability of adenosine to inhibit the influx of calcium through voltage sensitive calcium channels located on myenteric nerve endings of the small intestine. The findings support Ribeiro's hypothesis (1986) of the existence of A_3 receptors, mediating the inhibitory effects of adenosine, on nerve endings.

Gustaffson et al (1985) characterized the adenosine receptors involved in adenosine-induced regulation of cholinergic transmission in the guinea-pig ileum using selective adenosine receptor agonists for A_1 and A_2 types. The non-selective stable analog 2-CADO, the A_1 agonist R-PIA, the mixed A_1/A_2 agonist NECA were equipotent in inhibiting contractile responses to nerve stimulation, whereas S-PIA gave a similar response only at higher concentrations. The same potency profile was obtained for these agonists as inhibitors of Ach release which was measured by a gas chromatography and mass spectrometry. Manipulations which are known to enhance cAMP levels, enhanced the inhibitory effects of NECA but not R-PIA on contractions to nerve stimulation. It was also shown that NECA was the only agonist which inhibited contractions to direct muscle stimulation and the specific phosphodiesterase inhibitor ZK 62.711 enhanced these responses. The authors concluded that adenosine may inhibit the neuroeffector transmission, mainly by a prejunctional mechanism involving A_1 receptors and a supplementary activation of post-junctional A_2 receptors involving cAMP. In addition the evidence seem to suggest that a prejunctional inhibitory effect at high doses may be exerted via A_2 receptors.

Isolated myenteric varicosities were used to study the release of ATP (White & Leslie, 1981). The release of ATP was measured by monitoring the light produced when the released ATP reacted with firefly luciferin-luciferase which was present in the incubation medium. The characteristics of the ATP release measured were

consistent with a possible neurotransmitter function for ATP in the myenteric nervous system, but the possibility of co-release of ATP with another transmitter cannot be excluded. This work was followed by another report (Reese & Cooper, 1982) using the same preparation, which showed that low concentrations of adenosine or ATP inhibited the nicotinically-induced release of [^{3}H]-Ach from myenteric plexus synaptosomes. It was suggested from these studies that the receptor involved in these responses may be the P_1 or similar R site receptor. High concentrations of ATP caused a marked increase in release of loaded Ach, but the receptor nature of this interaction is not clear. Electrophysiological studies have attempted to elucidate the mechanism by which adenosine affects the electrical behavior of myenteric neurons of the guinea pig small intestine. Findings from such studies suggested that adenosine suppresses the catalytic activity of adenylate cyclase and consequently reduces the intraneuronal levels of cAMP by interacting with adenosine receptors on myenteric ganglion cells (Palmer et al, 1987).

Selectivity of agonists and antagonists for adenosine receptors

The most complete study on structure-activity relationships for xanthine antagonists at A_2 receptors has been that reported for fibroblasts (Bruns, 1981). This study led to the discovery of the potent 8-phenylxanthine series of antagonists. The use of A_1-receptor binding assays has now permitted more precise structure-activity data to be generated for several different xanthine

antagonists (Bruns et al, 1980; Bruns et al, 1983; Daly et al, 1983; Daly et al, 1985). These studies revealed that increasing the length of the 1,3-alkyl substituents of xanthines increases potency at A_1 receptors to a greater extent than at A_2 receptors. The addition of an 8-phenyl substituent to theophylline increases potency by some 25 to 35-fold at both A_1 and A_2 receptors, yielding a very potent but non-selective antagonist. In the 1,3-dipropylxanthine series, an 8-phenyl substituent enhances potency 50 fold at A_1-adenosine receptors but less than 10-fold at A_2 receptors, yielding a relatively selective and very potent A_1-adenosine receptor antagonist. Thus far, no modifications have led to any appreciable selectivity for A_2 receptors.

Bruns et al (1986) showed in ligand binding studies to rat striatal membranes,that the most selective agonist for A_2 receptors was the selective coronary vasodilator 2-phenylaminoadenosine (A_1, 560nM; A_2,120nM; ratio, 0.21). The most selective antagonist was 8-cyclopentyltheophylline (A_1, 11nM; A_2, 1400nM; ratio: 130), whereas alloxazine had slight A_2 selectivity (ratio: 0.52).

Ukena et al (1986) recently reported on the usefulness of [^{3}H]-XAC (xanthine amine congener, a functionalized derivative of 1,3-dipropyl-8-phenylxanthine) as an antagonist ligand for A_2 adenosine receptors of human platelets. It has been shown to inhibit NECA-induced stimulation of adenylate cyclase activity with a K_B of 24nM. [^{3}H]-XAC binding in platelets is the first example of labeling of A_2

adenosine receptors in which the potencies of adenosine agonists and antagonists in inhibiting binding are consistent with their potencies at these receptors in functional studies. This ligand is the first antagonist radioligand with high affinity for A_2 adenosine receptors.

Effects of adenosine on neurotransmitter release in the CNS.

Within the central nervous system, evidence suggests that the release of many transmitters is reduced by adenosine. Such transmitters include Ach (Vizi and Knoll, 1976; Jhamandas and Sawynok, 1976; Pedata et al, 1983), dopamine (Michaelis et al, 1979), glutamate (Dolphin and Archer,1983), serotonin (Harms et al,1979) and NA (Harms et al, 1978; Ebstein and Daly, 1982; Fredholm et al, 1983) and GABA (Harms et al, 1979; Hollins and Stone, 1980). The majority of such studies were carried out using brain slices or synaptosomes. However, with the exception of the synaptosomal experiments, such findings do not provide unequivocal evidence that the inhibition is a direct effect upon the nerve terminal. That is, inhibition of neural activity may result from a reduction in the number of times a nerve fires, rather than a decrease in the amount of transmitter released per impulse. These studies have demonstrated that adenosine has the ability to inhibit both excitatory and inhibitory transmitters in the brain. Whether these effects of adenosine may be extended to the myenteric nervous system of the small intestine is at present unclear.

In many instances, relatively high concentrations of adenosine agonists are needed to inhibit transmitter release and the inhibition is relatively weak. Concentrations of CHA less than 50 uM had no effect upon K^+-stimulated release of NA from guinea pig brain synaptosomes (Ebstein and Daly, 1982). In contrast, the EC_{50} for inhibition of synaptic responses by CHA in rat hippocampus is approx. 20nM (Dunwiddie et al., 1984) and the maximum response is almost 100 % inhibition. In some instances, however, the concentrations of agonists needed for inhibition are similar to those observed in electrophysiological studies (Harms et al, 1978; Dolphin and Archer, 1983). A possible explanation for these discrepancies is that the inhibition produced by nucleosides is dependent on the concentration of K^+ used to evoke release as was demonstrated for the release of dopamine (Michaelis et al, 1979).

The inhibitory effects of adenosine analogues on calcium-dependent K+-evoked release of labeled NA from guinea pig cerebral cortical and hippocampal synaptosomal preparations are most pronounced at low calcium concentrations (0.15mM) and can be prevented by increasing the calcium concentration in the medium (Ebstein & Daly, 1982). Adenosine inhibits the uptake of ^{45}Ca by synaptosomes depolarized by potassium (Kuroda, 1983).

It is worth noting that the inhibitory profile of adenosine analogs on K+-depolarization and electrical stimulation of transmitter release from central and peripheral tissues appear to be different

(Rebeiro & Sebastio, 1986). The reason for this difference is not clear at the present time.

Identification of adenosinergic neurons.

Adenosine is a small molecule and, therefore, closer to the classical neurotransmitters, than to the neuropeptides. Unlike other neurotransmitters, no evidence exists for its storage in nerve or other cells or for its exocytotic release although some progress has recently been made in defining adenosinergic neurons. The use of specific antisera to adenosine (Snyder et al, 1984) has yielded preliminary data suggesting that the distribution of immunoreactive neurons, e.g. in hippocampal pyramidal cells, parallels the distribution of A_1 receptors and 5'-nucleotidase activity. Such studies have not been carried out on small intestinal tissue. The specificity of the adenosine immunoreactivity for 'adenosinergic' neurons is still unclear. A transport mechanism of the fascilitated diffusion type has been postulated either for adenosine release or its uptake (Arch and Newsholme, 1978). Adenosine derives from cytosolic precursors (see Daly,1982).

Correlation of binding and functional effects of adenosine.

In excitable tissues, only a few studies have correlated binding and functional effects of adenosine analogs. Kuroda et al (1976) showed

a good correlation between physiological responses and cAMP changes which would suggest the involvement of an A_2 receptor. Dunwiddie & Fredholm (1984) showed that the inhibitory effects of adenosine analogs on synaptic transmission are mediated via a receptor with a pharmacological profile different from the adenosine receptor linked to AC.

An excellent correlation was found between the affinities of selective adenosine receptor analogs for binding sites labeled by [^{3}H]- CHA and their potencies in depressing synaptic transmission (Reddington et al, 1982, 1985), which indicated that CHA binds to adenosine receptors which are similar to those which are involved in the inhibition of transmitter release.

Although the existence of several different adenosine receptor subtypes have been clearly established, it is often difficult to identify which receptor subtype mediates a specific biochemical or physiological response. Selective alkylxanthine antagonists discriminate somewhat between A_1 and A_2 receptor subtypes and therefore classical pharmacological techniques such as Schild analysis may be of some use in discriminating between receptor subtypes. However, to date, the most useful criterion in identifying adenosine receptor types is determination of the relative potencies of several selective analogs in modulating transmitter release. The potencies of a variety of analogs have been determined in physiological, biochemical and behavioral systems (Daly, 1983).

Distribution of substance P in the small intestine.

The putative neurotransmitter or neuromodulator role of substance P in the mammalian nervous system has relied in many cases on its immunohistochemical localization in neuronal structures and its identification by specific radioimmunoassay (RIA) in extracts of various neural tissues. Given that two additional mammalian tachykinins exist in neural structures, the possibility exists that identified substance P is really one of the other tachykinins. As most antisera raised against substance P are polyclonal and directed against the C-terminal portion of the tachykinin, the portion of the tachykinins most conserved in this peptide family, in many cases they do not discriminate well between the various mammalian tachykinins.

The tachykinins alpha-neurokinin and substance P may be colocalized, co-synthesized, and / or co-released from several brain regions (Dalsgaard et al, 1985; Lindefors et al, 1985, Lee et al, 1986). The regional distribution of beta-neurokinin appears to be quite distinct from that of alpha-neurokinin and substance P (Ogawa et al, 1985). Several studies have shown that a calcium-dependent release of tachykinin-like immunoreactivity (TKLI) can be evoked from synaptosomes (Lindefors et al, 1985). The most popular mechanism proposed for inactivation of the tachykinins upon release, is proteolytic degradation and candidate enzymes have been suggested (Krause, 1985). Development of specific antisera, radioligands and antagonists for receptors recognized by these mammalian tachykinins

will permit elucidation of the physiological role(s) played by these neuropeptides.

The morphology and distribution of SP containing neurons has been most extensively investigated in the gastrointestinal tract of the guinea pig and rat (Bartho & Holzer, 1985) and these neurons form a very dense network in the myenteric plexus as well as other layers of the gut. The nerve cell bodies of the enteric neurons containing SP are present in the myenteric and submucosal plexuses. The distribution of SPLI in the different layers of the gut has been described in detail (Jessen et al, 1980; Costa et al, 1980, 1981). SPLI has been shown to be concentrated in the intestinal muscle layer including the myenteric plexus.

Co-existence of choline acetyltransferase and SP was demonstrated by Furness et al (1984) in the submucosa of the guinea pig ileum. The neuropeptide met-enkephalin and the amine 5-HT have also been shown to coexist with SP in enteric neurons (Domoto et al, 1984; Legay et al, 1984).

Release of substance P from myenteric neurons of the small intestine.

A great deal of evidence exists which indicates that SP is released from myenteric neurons of the small intestine. It has been well documented that neuronally mediated ,non-cholinergic longitudinal

contractions of the guinea pig ileum are mediated by SP or a related tachykinin released from intrinsic nerves (Franco et al, 1979). Electrical field stimulation or a rise in K^+ concentration was shown to increase release of SPLI from myenteric nerves which was dependent on the extracellular calcium concentration. The release of SPLI was dependent on both the frequency of stimulation and the K^+ concentration. Gel-exclusion chromatography established that the SPLI released from the myenteric plexus by depolarizing stimuli is apparently identical to authentic SP (Holzer, 1983, 1984). Baron et al (1983) and Gintzler et al (1983) showed that the amount of SPLI released from myenteric nerves of the guinea pig ileum was not linearly related to the frequency of stimulation (0.5-40Hz). Together with the finding that tetrodotoxin only partially reduced the SPLI release caused by 20 Hz stimulation, these findings allowed Baron et al (1983) to suggest that SPLI is released from more than one type of neuron, the different types being recruited by different stimulation frequencies and having different sensitivities to tetrodotoxin. Since the SPLI measured by Baron et al (1983) was not chemically characterized, it is not clear whether all the SPLI represents authentic SP or one of the other tachykinins. Acetylcholine and CCK8 were both shown to significantly increase the release of SPLI from the myenteric plexus (Holzer, 1984). The non-cholinergic contractile response to CCK8 is reduced to a variable degree by the ganglionic blocking drug hexamethonium (Bartho & Holzer, 1985).

Direct evidence for the release of acetylcholine by SP has also been presented by using the loaded acetylcholine technique (Fosbraey & Johnson, 1980) and evidence supporting that opioids reduce the release of enteric SP has also been reported (Bartho et al, 1982; Scalisi, 1982). The view that exogenous and endogenous opioid peptides inhibit the release of enteric SP is also supported by immunohistochemical data presented by Holzer (1984). This author showed that a met-enkephalin analogue FK 33-824 significantly reduced the stimulus-induced release of SPLI from the myenteric plexus of the guinea-pig ileum. This effect was blocked by naloxone. By itself, naloxone significantly enhanced the stimulus-induced release of SPLI (Holzer,1984).

It appears that drugs or procedures that influence SP release also influence the release of Ach from enteric nerves. It has been shown that opiates and alpha-adrenergic agonists inhibit both Ach and SP release whereas CCK8, neurotensin and ganglionic stimulants stimulate release of both. It has therefore been suggested that receptors for these drugs may be present on both SP and cholinergic neurons (Bartho & Holzer, 1985).

Inactivation of substance P.

When SP is released from the myenteric plexus of the guinea pig ileum it rapidly loses its immunoreactivity, an indication of its rapid enzymatic degradation (Holzer, 1984). From the results of Watson (1983) it would appear that the half-life of [^{3}H]-SP in the

extracellular space of the guinea pig ileum is less than 30 s. Whether SP released from myenteric nerves is degraded by specific peptidases under physiological conditions is still unclear.

The available evidence suggests that nucleosides and/or ATP as well as substance P, play a physiological role in neuromodulation and / or neurotransmission in the enteric nervous system. The role of the newly identified mammalian tachykinins alpha-neurokinin and beta-neurokinin in neurotransmission is still not clear and, in the myenteric nervous system, no functional evidence exists for their release. A great deal of evidence supports the existence of heterogeneity of adenosine receptors in both the central and peripheral nervous systems. The role of adenosine on the modulation of other excitatory neurotransmitter-like substances in the enteric nervous system is still not well understood, nor is the precise mechanism by which adenosine mediates inhibition of transmitter release.

METHODS

GENERAL SYNOPSIS FOR THE METHODOLOGY

Each section in the methods is presented separately and contains: specific details of each procedure, chemicals and sources, and method of statistical analysis (see also results section)

For statistical analysis, a $p < .05$ was accepted as being significant.

A separate page at the front of the thesis lists abbreviated terms used frequently throughout the thesis.

Figures pertaining to the methods section are lettered A through E and are located at the end of the section. Figures pertaining to the results section are numbered 1 through 56 and are located at the end of the results section.

A) ISOLATION OF PARTIALLY PURIFIED-INTACT MYENTERIC VARICOSITIES FOR BINDING STUDIES WITH TRITIATED NUCLEOSIDE ANALOGS.

i) ISOLATION OF PV FOR BINDING ASSAYS

Male or female guinea pigs weighing from 300 to 400 g were used and each experiment required tissue derived from 12 guinea pigs. Isolated myenteric varicosities were prepared according to the methods developed by Jonakait et al.(1979) and Briggs and Cooper (1981) as modified by White and Leslie (1982). Some minor modifications were also incorporated. All steps were carried out at 0°C - 4°C.

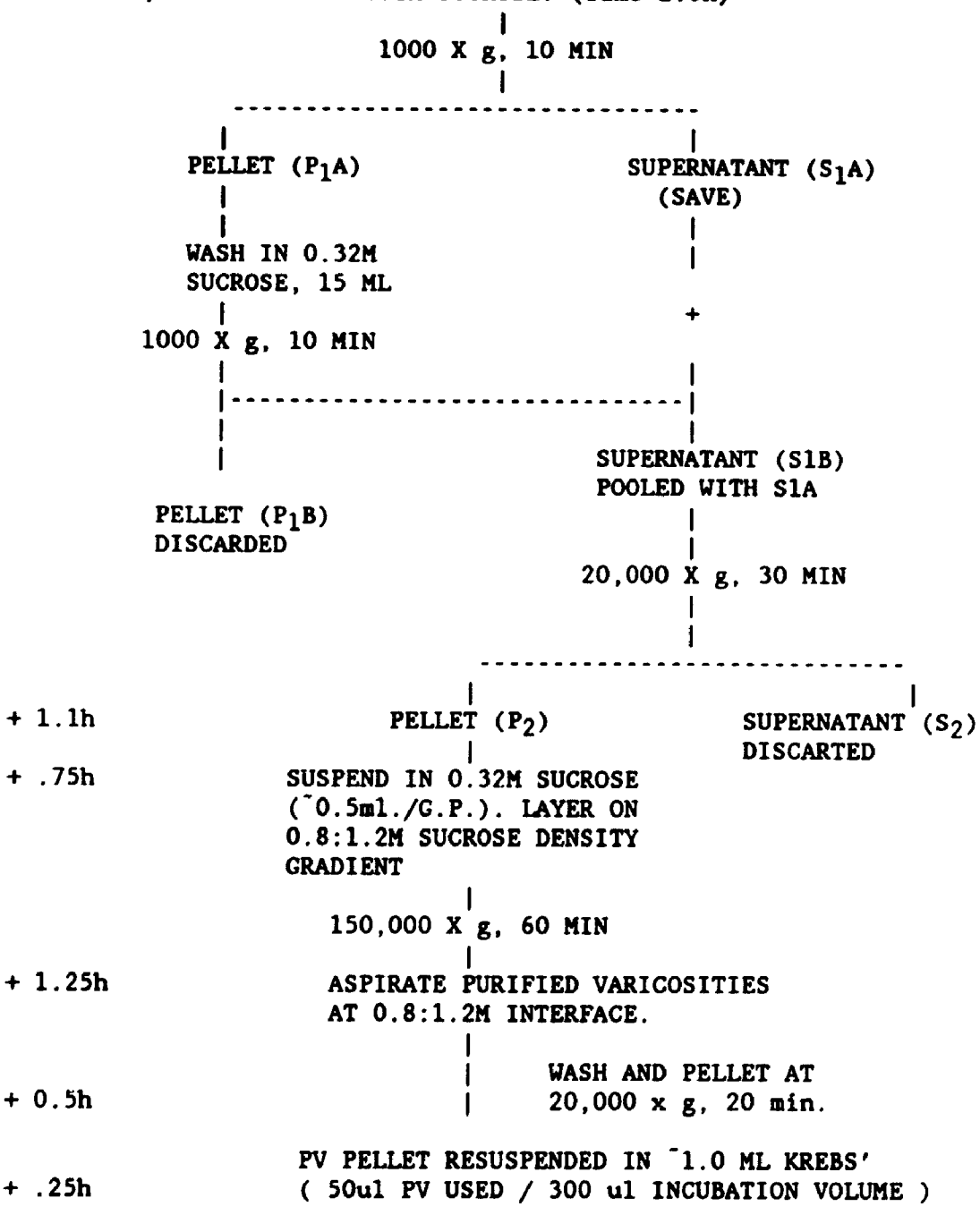
The P_2 pellet was resuspended to a total volume of 6.0 ml in 0.32M sucrose and 1.0 ml aliquots were layered onto each of six discontinuous sucrose density gradients consisting of 2.0 ml of 0.8M sucrose layered on 1 ml of 1.2M sucrose. After ultracentrifugation at 150,000 X g for 1.0 h at 4 C using an SW 50.1 rotor on a Beckman L4 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA), the PV fraction was collected at the 0.8M:1.2M sucrose interface by aspiration with a glass pipette. When necessary the mitochondrial pellet at the bottom of the 1.2M sucrose layer was recovered from each gradient tube by resuspending in 1.0 ml of Krebs' solution. The PV fraction was washed with 15 ml of Krebs'solution (pH 7.4) and centrifuged at 20,000 X g for 20 min to yield a pellet. The pellet was resuspended in convenient volumes of Krebs' solution (~1.0 ml) in order to carry out binding assays. A schematic diagram of the isolation of PV for binding assays is presented in schematic diagram 1. (overleaf)

iii) Occluded LDH AND LOWRY PROTEIN ASSAYS

Occluded lactate dehydrogenase (LDH), as a marker of cytoplasm enclosed within a cell membrane, was routinely determined on the PV fraction. Thirty microliters of PV suspension or 100 ul of mitochondrial suspension was used in the determination, which was carried out according to Whittaker and Barker (1972) and White and Leslie (1982). Protein concentration was determined by the Hartree (1972) modification of the method of Lowry et al.(1951).

PREPAPATION OF PURIFIED MYENTERIC VARICOSITIES (PV) FOR BINDING ASSAYS

ii) SCHEMATIC DIAGRAM #1.



iv) ELECTRON MICROSCOPY

Aliquots of the purified varicosity fraction (25-50ul) in Krebs's solution were added to fixative solution and fixed for 2 h at room temperature. The fixative contained 1.5% glutaraldehyde and 1% paraformaldehyde in 0.1M sodium cacodylate buffer at pH 7.3. After fixing, the varicosities were centrifuged to a pellet and left in fixative for a further 2 h. The pellet was then washed twice in 0.2M cacodylate buffer and the preparation postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1.5 h. After washing in 0.1M cacodylate buffer the preparation was dehydrated in an ascending series of ethanol solutions to 70% ethanol, stained en bloc with saturated uranyl acetate in 70% ethanol for 1 h and further dehydrated in ethanol and two changes of propylene oxide. After embedding in Spurr Resin, thin sections were obtained using a diamond knife, the grids stained with uranyl acetate and lead citrate, and the sections were viewed in a JEOL model JEM-100CX-II electron microscope.

v) COMPETITION EXPERIMENTS INVOLVING TRITIATED ADENOSINE RECEPTOR ANALOGS

a) Methodology

The measurement of displacement of [^{3}H]R-PIA, [^{3}H]NECA and [^{3}H]CHA binding to the PV fraction was performed using a rapid centrifugation technique. Fifty microliters of PV suspension (approx. 100ug protein) were added in 1.5 ml microcentrifuge tubes to 300 ul of Krebs' solution containing either no unlabeled competitor or various concentrations of unlabeled competitor (eg. adenosine receptor agonist or antagonist). The tubes contained 5nM [^{3}H]R-PIA, 20nM [^{3}H]NECA or 20nM [^{3}H]CHA as the radioligand and 2.0 units of adenosine deaminase per ml to degrade endogenous adenosine that may be present in the PV. After incubation at 22 C for 12 min tubes were centrifuged at 12,800 x g for 90 sec, in a Savant high speed centrifuge (Savant Instruments Inc., Hicksville, NY), and the supernatant containing the unbound radioactivity was removed and discarted. Pellets were surface washed with 1.25 ml of ice-cold Krebs' solution and, after recentrifugation and aspiration of the wash, were dissolved in 500 ul of 0.1 M NaOH. Fifty microliters of this hydrolysate were removed for estimation of individual protein concentrations and the remainder transferred complete with tube to 6.0 ml of Beckman Ready Solve EP scintillation fluid and counted in a liquid scintillation spectrometer.

b) Analysis of competition data

Competition curves were constructed using the pooled data obtained from three to five separate experiments unless otherwise specified in the text. The concentration of competitor was plotted against the percentage of specific binding using the maximum displacement of each radioligand by 100 uM of its unlabeled counterpart as 100%

specific binding.

Displacement curves were calculated using the nonlinear curve-fitting technique described by DeLean et al. (1978). This was achieved using the ALLFIT program described elsewhere in the methods. The IC_{50} values for the competitors was obtained from the fitted curves. S.E.M. were also calculated for all the data points on the displacement curves.

IC_{50} values obtained for various adenosine analogs as displacers of labeled R-PIA and NECA binding were compared to their EC_{50} values as inhibitors of acetylcholine release at the stimulated ileum preparation. The correlation between IC_{50}'s and EC_{50}'s was presented as a regression line through the points and the 95% confidence interval for the slope of the regression line was calculated (Goldstein,1964) [The slope is not significantly different from unity if the confidence interval includes 1.0].

vi) Drugs and Chemicals.

a) Nucleoside analogs and related compounds

R-PIA and CHA were obtained from Boehinger Mannheim Canada (Montreal, Quebec, Canada) and NECA was kindly donated by Abbott Laboratories (North Chicago, IL). PACPX was obtained from Research Biochemicals Inc.(Wayland,MA). DPSPX, MCPA and CPA were kindly donated by R.A.Olsson, University of South Florida. Adenosine deaminase (Type VIII) was obtained from Sigma Chemical Co. (St. Lois,Mo.)and was dialyzed against 0.1M Tris buffer,pH 7.5, before use. 2-chloroadenosine was obtained from Sigma. Dimethyl sulfoxide was from J. T. Baker Chemical Co. (Phillipsburg,NJ). Purine riboside and inosine were supplied by Aldrich Chemical Co. (Milwaukee, WI). Adenosine-N^1-oxide was obtained from U.S. Biochemical, Cleveland, OH.

b) Labeled nucleosides

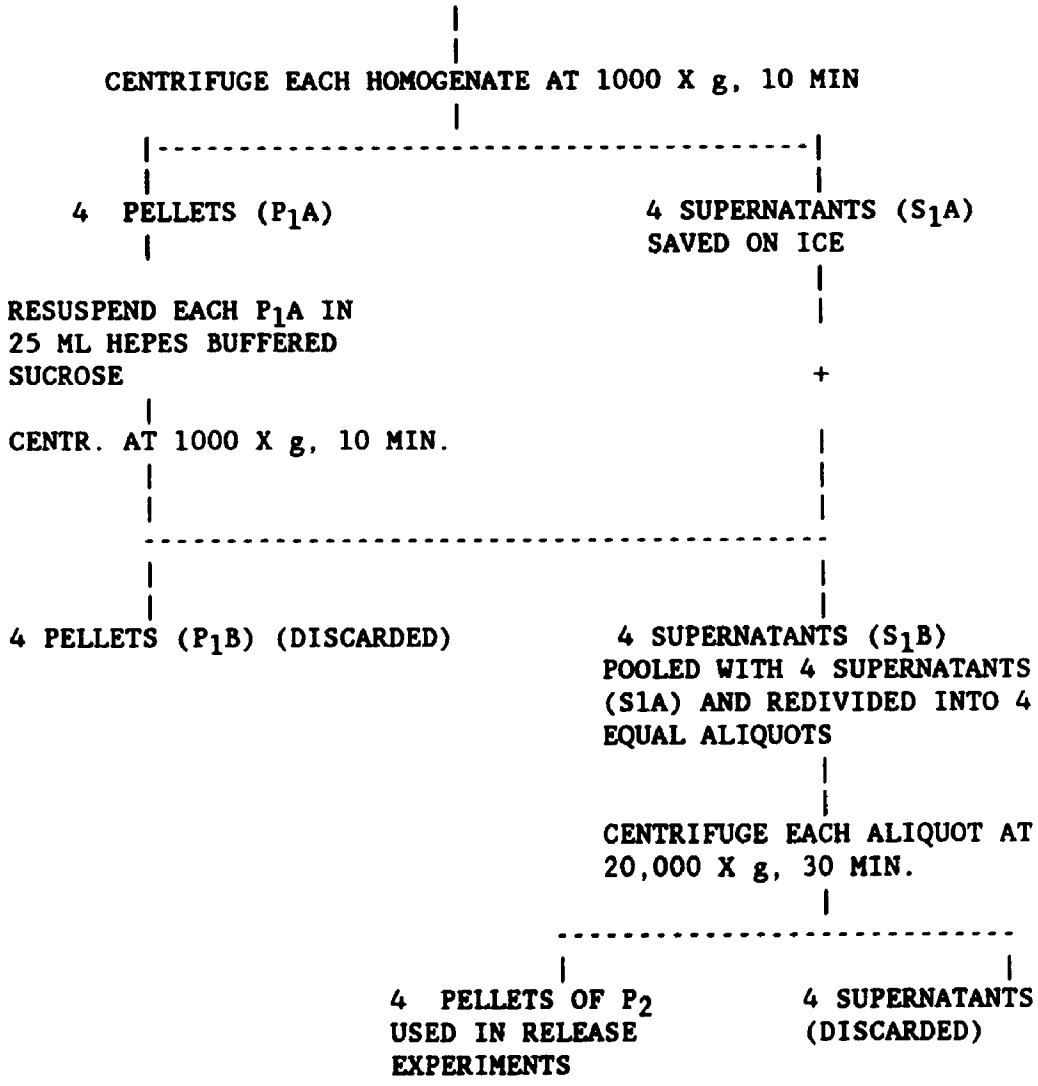
Labeled nucleoside analogs used in these studies were obtained from Amersham Canada Ltd. (Oakville, Ontario, Canada). (-)-N^6-R-[G-^{3}H]phenylisopropyladenosine was supplied with a specific activity of 42 to 49 Ci/mmol; 5'-N-ethylcarboxamide[8(n)-^{3}H]adenosine and N^6-[adenine-2,8,^{3}H]cyclohexyladenosine with specific activities between 21 and 31 Ci/mmol. The labeled nucleosides were dried in aliquots containing 5 to 10 uCi using a Savant vacuum centrifuge and used immediately.

B. PREPAPATION OF CRUDE MYENTERIC VARICOSITIES (P_2) USED IN STUDIES MEASURING RELEASE OF NEUROPEPTIDES.

i) SCHEMATIC DIAGRAM #2

ISOLATION OF P_2

The pooled LM-MP stripped from the ilea of 8 guinea pigs was minced, weighed and divided into 4 equal amounts. Each aliquot was homogenized with a teflon glass homogenizer in 25 ml of isolation buffer as described earlier. The composition of the buffer was as follows: 10mM HEPES; .31M SUCROSE; 50uM EGTA; pH to 7.2 with 10N NaOH.



ii) Resuspension and equilibration of each P_2 pellet

Each of the 4 P_2 pellets was resuspended in 5.5 ml of ice cold Locke's solution of the following composition (millimolar): NaCl,140; KCl,5; $NaHCO_3$,5; D-glu,10; $MgCl_2$,1; $CaCl_2$,2.54; HEPES,10; pHed TO 7.2 with 10N NaOH and then .05% BSA was added to buffer to prevent binding of released peptides to incubation tubes. Unless otherwise specified, each synaptosomal suspension was equilibrated at 37 C for 60 to 75 min. Then 5mM bacitracin was added to the P2 suspensions in order to inhibit proteolytic enzymes. A 6 min equilibration with bacitracin was allowed before carrying-out the release reaction.

iii) Incubation of P_2 suspensions with agonists

Agents were added to 1.5 ml polypropylene microcentrifuge tubes in 500 ul aliquots from appropriate stock solutions made up in Locke's buffer. A 500 ul aliquot of P_2 suspension was quickly added to the tubes to initiate the reaction. The reaction was allowed to proceed for 3 min at 37 C at which time the samples were centrifuged at 12,500 X g for 1.0 min using a Savant high speed centrifuge (Savant instruments Inc.,Hicksville,NY). The supernatants containing the released neuropeptides were immediately removed with Pasteur pipettes and boiled at 98 C for 6 min to destroy any remaining proteolytic enzyme activity and the pellets were discarded. The supernatants were cooled to room temperature and 0.4 % BSA was added to prevent binding of peptides to walls of tubes. The sample supernatants were stored at -86 C until assay.

iv) Use of the P_2 suspension to determine dose-effect relationships of agonists.

Each 5.5 ml P_2 suspension was sufficient tissue for 10 separate incubations, each incubation containing a different dose of the agonist. Therefore, a 10 point dose-response curve could be constructed from RIA data obtained from each P_2 suspension derived from the ilea of 2 guinea pigs. From a total of 8 guinea pigs used in each experiment (4 P_2's), a total of 4 dose-response curves could be constructed, each containing a different agonist and/or representing a different condition. This protocol offered the advantage of being able to compare dose-effect relationships under 4 different conditions within the same experiment [The pooling of all S_{1A}'s and S_{1B}'s and their subsequent division into equal aliquots insured that the 4 resulting P_2's are homogeneous and contain the same concentration of varicosities].

v) Log-logit transformation of the RIA standard curves.

RIA standard curves were analysed using log-logit transformations. The log [(B/F)/(K-B/F)] was plotted against the log [standard] which yielded a straight line function. The individual linear

regression points were compared with their transformed counterparts. Point by point error differences between the respective lines having values < 10% were deemed acceptable. Those points with < 10% error were included in the final log-logit transformed line used to estimate the immunoreactivity in the samples. K is a variable which was manipulated to give a log-logit line with a correlation coefficient approaching 1.0 for all the standard curves.

vi) Atomic Absorption Spectrometry

The divalent cation concentrations for Ca^{++} and Mg^{++} were measured using atomic absorption spectrometry. The K^{+} concentrations were measured by atomic emission. Standard solutions of $CaCl_2$, $MgSO_4$ and KCl were prepared in d.H_2O.

For atomic absorption, appropriate hollow cathode lamps were used giving line spectra for Ca^{++} at 422.7nm and for Mg^{++} at 285.2nm. The flame used was acetylene:air with a reducing stoichiometry. Standards and samples were aspirated into the flame in succession. The absorbance values of the standards were used to construct standard curves and sample concentrations (ppm) were extrapolated from these curves. Correction for molecular absorption was obtained using a deuterium lamp.

For emission spectroscopy (e.g.K^{+}), the monochromator was set at the line frequency for K^{+} (766.5nm) and standards and unknowns were aspirated into the flame in a similar manner.

vii) Membrane Potential (V_m) Determination

The membrane potential of the myenteric synaptosomes was determined by the TPP+ cation method (Robert et al. 1978, Deutsch and Rafalowska, 1979, Scott and Nicholls, 1980).

viii) Dissociation curve for [^{3}H]NECA at PV

The PV pellet obtained from the ilea of 12 guinea pigs was resuspended in a total volume of 550 ul Krebs and equilibrated at 22 C for 30 minutes. The tissue was then mixed with 2 u/ml Adenosine Deaminase (VI) and incubated for 10 min. Then 1.0e-^{7}M ^{3}HNECA (21Ci/mmole) for a further 10 minutes. To start the dissociation reaction, 50ul aliquots of the PV suspension (~125ug PV protein) were injected into a 3.0 ml volume of Krebs (pH 7.4) in a 15ml pyrex test tubes and the reaction allowed to proceed for various time intervals (0, 1, 2, 5, 10, 15 min). Termination of the reaction was accomplished by centrifugation (4 C) at 20,000 g for 5 min. The amount of radioactivity bound to the pellet was measured in a scintillation counter as before.

C) CHARACTERIZATION OF CONTENT AND CHROMATOGRAPHIC FORMS OF NEUROPEPTIDES IN BOTH CRUDE (P_2) AND PURIFIED NERVE VARICOSITIES (PV) PREPARED FROM GUINEA PIG LMMP.

i) Isolation and preparation of aliquots of varicosities

Four batches of crude varicosities (p_2) were prepared from myenteric plexus of guinea pig ileum using homogenization and centrifugation as described above. Each separate homogenate was derived from the ilea of 2 guinea pigs. Each P_2 pellet was resuspended into 5.0 ml of Krebs solution (which had been gassed for 15 min with 95% O_2/5% CO_2 and adjusted to a pH of 7.4) and equilibrated for 60 minutes. The P_2 suspensions were divided into 1.0 ml aliquots. In addition, seven batches of partially purified varicosities (PV) were prepared from myenteric plexus of guinea pig ileum (12 animals per batch) by resuspending P_2 pellets in 0.32M sucrose, dividing into 1.0 ml aliquots and layering onto discontinuous density gradients. Following ultracentrifugation, purified varicosities aspirated from the 0.8M/1.2M interface, were washed, resuspended in Krebs solution and equilibrated for 60 min. The PV suspensions were divided into aliquots of 400 ul in Eppendorf tubes. For further details of the isolation procedure of the P_2 and PV preparations refer to Methods section A, part i and section B, part i respectively.

Following the equilibration period (this is the period allowed for P_2 prior to addition of releasing agent) both P_2 and PV aliquots were subjected to heat inactivation at 98 C for 10 min in a heating block to denature proteolytic enzymes. The inactivated varicosity suspensions were centrifuged at 12,500 g for 3 min and the supernatants were removed and stored at - 86 C until performance of the specific RIA's or HPLC chromatography.

HPLC analysis was carried out in collaboration with Dr. T.J. McDonald, Dept.of Medicine, University of Western Ont. London. The protein content of the synaptosomal suspensions was determined by the Hartree (1972) modification of the Lowry (1951) method using bovine serum albumin as the standard. Portions of suspensions from three PV preparations were aliquoted before centrifugation, sufficient glacial acetic acid was added to make the solution 0.5M in acetic acid, the suspensions were then vigorously mixed for 20 min followed by centrifugation at 4 C. Before the RIA was carried-out,the acidified supernatants were lyophilized to remove the acetic acid and reconstituted with the appropriate buffer or sufficiently diluted with appropriate buffer to neutralize the acetic acid. RIA for neuropeptides (vide infra) was performed on serial dilutions of all aliquots. Aliquots of the preparations were subjected to reverse-phase HPLC (vide infra) with eluates being collected in 2 min fractions, diluted with freshly boiled 0.2% (w/v) peptone Type I (Sigma Chemicals,St. Louis,MO) solution, lyophilized, and each eluate fraction was reconstituted with appropriate buffers and then subjected to specific RIA for individual peptides.
Eluate fractions of P_2 aliquots were subjected to RIA for both

alpha-neurokinin and SP- like-immunoreactivities. Fractions of PV suspensions were only subjected to RIA for SPLI, since only the SP RIA was available in this laboratory at the time these experiments were carried-out.

ii) Reverse-Phase HPLC

Reverse-phase HPLC was performed using a Waters instrument consisting of a reverse-phase micro-Bondapak C-18 column (3.9 x 300 mm), a U6K injector, two M 6000 A pumps, a 660 solvent programmer and a 450 model UV detector. Peptide peaks were detected by monitoring the absorbance at 215 nm. Solvent systems were 0.1% trifluoroacetic acid in water (A) and acetonitrile (B), and the flow rate was 1 ml/min.

In the first set of experiments designed to screen for the overall content and elution positions of immunmoreactive neuropeptide entities, 150 ul aliquots of PV preparations (1.6-2.4mg/ml) were applied to the column and eluted with a steep linear gradient from 15 to 55% solvent B over 90 min. A second set of experiments were performed under conditions of higher resolution, in an effort to document with more precision the elution positions of selected neuropeptide immunoreactivities. Aliquots of the same PV preparations were injected in volumes of 300 ul and eluted with a 'shallow' linear gradient of 24 to 28% solvent B over 60 min. Two min fractions were collected as described above. In another set of experiments, 1000ul aliquots of P_2 suspensions (0.7 - 1.0 mg protein/ml) were applied to the column and eluted with the 'shallow' gradient (24-28% solvent B) over 90 min and two minute fractions were collected.

For use in RIA and for HPLC calibration, the synthetic analogues of galanin, porcine and guinea pig vasoactive intestinal polypeptide (VIP), substance P (SP) and its C-terminal fragments SP(2-11), SP(3-11), SP(4-11), SP(5-11), SP(6-11), SP(7-11), SP(8-11), alpha-neurokinin and beta-neurokinin, Leu-enkephalin and Met-enkephalin, phosphate acceptor peptide, motilin, PHI, secretin, dynorphin, neurotensin, gonadoliberin, porcine gastrin-releasing polypeptide (GRP[18-27]), and physalaemin were purchased from Peninsula Laboratories (Balmont,CA). The elution positions of standard synthetic peptides on the reverse-phase HPLC were obtained by injecting individual synthetic peptides and monitoring their UV absorbance elution pattern at 215nm. Calibration runs on HPLC with the oxidized forms of substance P, alpha- and beta-neurokinin, GRP(1-27), GRP(18-27), Met-enkephalin and VIP were performed after oxidation of peptides for 15 and 60 min incubation times with 1% hydrogen peroxide in water (v/v) at room temperature.

After all calibration runs and before chromatography of the PV and P_2 synaptosomal lysates, the reverse-phase column, tubing and injection port were thoroughly washed and, after injection of 150 - 1000 ul solvent A, at least 3 successive blank chromatography runs

were performed under 'steep' and 'shallow' gradient elution conditions as described and the collected eluate fractions subjected to all RIA's. These blank runs did not contain detectable amounts of any of the neuropeptides.

D) SPECIFIC RADIOIMMUNOASSAYS FOR QUANTITATING INDIVIDUAL PEPTIDE IMMUNOREACTIVE ENTITIES

In general, the specific radioimmunoassays followed similar protocols. Briefly, incubation mixtures contained tracer alone (blanks for standards), tracer + sample (sample blanks) and tracer + unknown sample + antiserum were incubated for periods ranging from 24 h to 5 days at 4 C. Separation of bound from free tracer in the samples was accomplished using the dextran-coated charcoal method. The ice-cold charcoal solution was quickly mixed with the contents of each sample tube, vortexed and immediately centrifuged at 2,500 x g for 10 min using a standard bench top centrifuge. The charcoal-bound radioactivity in the pellet represented the free tracer not bound to the antiserum during the incubation. The supernatant represented the antiserum-bound tracer. Both bound and free tracer was counted using a Beckman gamma counter. The quantitation of neuropeptide immunoreactivity in the samples involved duplicate determinations at several different dilutions of each sample.

i) Tachykinins

Radi immunoassay for substance P was performed using antiserum K-25 (kind gift of Dr. G. Nilson, School of Veterinary Medicine, Uppsala, Sweden). Details of antiserum characterization and RIA procedures have been described previously (Brodin et al,1981). The K-25 antiserum detects 1.5 fmol substance P per assay tube (10% drop from initial binding) under conditions employed in this laboratory.

The buffer system which was used in both the alpha-neurokinin and SP RIA's contained: Na_2HPO_4, 0.5M; NaN_3, 0.05M; EDTA, 0.01M; pH 7.4; 0.8% BSA.

ii) cross-reactivity of tachykinins

SP cross-reacted with alpha-neurokinin at 0.3 % compared to substance P (100%).

iii) Enkephalins

Radioimmunoassay for enkephalin-like immunoreactivity (ENKLI) was performed using an antiserum raised against met-enkephalin (kindly donated by Dr. C. Sundberg, Bowman-Gray School of Medicine, Winston-Salem, NC) but which cross-reacted with Leu-enkephalin 2 times more potently than Met-enkephalin. The antiserum characteristics and assay procedure have been described in detail by Sundberg and Dunlap (1986). In this laboratory, the antiserum detects 20 fmol of Met-

enkephalin per assay tube (10% drop from initial binding), with synthetic Met-enkephalin as standard and radiolabelled Met-enkephalin purchased from Immunonuclear Corp. (Stillwater,MN).

Leu-enkephalin- and met-enkephalin-specific antisera were also purchased from Immunonuclear Corp. (Stillwater,MN) and have the following specifications: the Met-enkephalin antiserum cross-reacts with Leu-enkephalin at 2.8% compared to Met-enkephalin (100%) and the Leu-enkephalin antiserum cross-reacts with Met-enkephalin at 1.1% compared to Leu-enkephalin (100%). Under conditions used in this laboratory, the Leu-enkephalin RIA system detects 1.5 fmol Leu-enkephalin per assay tube and the Met- enkephalin RIA system detects 20 fmol Met-enkephalin per assay tube (10% drop from initial binding). Synthetic Leu- and Met-enkephalin used as standards and radiolabeled Leu-and Met-enkephalins, were purchased from Immunonuclear Corp. (Stillwater,MN), and used in the respective Leu- and Met-enkephalin RIA's using assay contitions suggested by the manufacturer. These antisera were used to measure the content of leu and met-enkephalin-LI's in both the PV and P_2 preparations. The LE antiserum was also used to measure the release of LE from P_2.

The buffer system used in the ME RIA consisted of .01M Na_2HPO_4 and .05% NaN_3. The pH was adjusted to 8.0 with 5M phosphoric acid and then 0.2% BSA was added to the buffer. The North Carolina antiserum was used in a final dilution of 1:12,000. The standard stock solution was 100,000 fmol/ml and serial dilutions ranging from 12.5 to 12,800 fmol/ml were used to construct the standard curve for MELI. The sample, standards, tracer and antiserum were added in 0.1 ml volumes and the total incubation volume was only 0.4 ml. The incubation time was 24 h at 4 C. The composition of the dextran-coated charcoal employed in the separation of bound and free tracer was as follows: 0.2% charcoal and .02% dextran; 1.0 ml of ice-cold charcoal buffer and 0.1ml of pig plasma were added to the incubation tubes.

The buffer system used in the LE RIA contained 0.1M boric acid, adjusted to pH 8.2 and then 0.1% peptone was added to the buffer. The LE antiserum was used in a final dilution of 1:9,000. The standard stock was 100,000 fmol/ml and serial dilutions ranging from 6.25 to 3200 fmol/ml were used in obtaining the standard curve. The incubation volume was 0.6 ml. Following a 24 h incubation period at 4 C, the bound and free tracer were separated by the charcoal method which consisted of 0.5% NORITA charcoal and .05% dextran; 1.0 ml ice-cold charcoal buffer and 0.1ml of pig plasma were added to the tubes and vortexed. Samples were centrifuged as reported above.

iv) Peptides not present in the enteric nervous system

a) Gastrin

Gastrin-like immunoreactivity in the PV preparation was performed using antiserum 2604 (a gift of Dr.J. Rehfeld, Copenhagen, Denmark)

as described by Rehfeld et al. (1972) with the only difference being that bound and free tracer were separated by use of charcoal-dextran mixture. This RIA system detects 0.5 pg gastrin-17 per assay tube (10% drop from initial binding).

b)PYY

RIA for PYY-like immunoreactivity in the PV preparation was performed using an antiserum (LR-57) obtained from a rabbit after multiple intradermal injections) in Freund's adjuvant; bleedings were obtained 7-10 days post injection. The LR-57 is used in a final dilution of 1:40,000 and detects 4 fmol of PYY per assay tube (10% drop from initial binding). It does not cross-react with any structurally unrelated GEP peptide tested and has no cross-reaction with the structurally related pancreatic polypeptide and only a minor cross-reaction (30% drop from initial binding) with NPY was prepared by the chloramine-T method (Hunter & Greenwood,1962), subjected to a preliminary purification on a Sep-Pak C-18 cartridge followed by chromatography on a carboxymethylcellulose column (0.9 x 28 cm) equilibrated and eluted under isocratic conditions with a 0.0225 M phosphate buffer pH 6.5 containing 2% BSA (W/V). Fractions from the descending portion of the single major peak of radioactivity (eluting at ca. 11 column volumes) consistently contain radiolabelled PYY with the best antibody and receptor binding characteristics.

v) GASTRIN RELEASING POLYPEPTIDE

RIA for the gastrin-releasing polypeptide (GRP) was performed using an antiserum (LR-16) raised in a rabbit subjected to multiple intradermal injections of synthetic GRP (85 ug initial, 40 ug subsequent injections) in Freund's adjuvant with bleedings obtained 7-10 days postinjection. This antiserum is used in a final dilution of 1:60,000 for RIA and can detect 1 fmol GRP per assay tube (10% drop from initial binding). It is C-terminally directed and reacts equally well with bombesin or GRP and binds equally well with radiolabelled tyrosine-4-bombesin (Peninsula, Belmont,CA) or hydroxyl-propyl-phenyl-GRP-(18-27) (HPP-GRP-(1827)) (a kind gift of Dr. N. Yanaihara, Shizuko University, Japan). Either tyrosine-4-bombesin or HPP-GRP-(18-27) was iodinated with chloramine-T (Hunter and Greenwood,1962) subjected to a preliminary purification on a Sep-Pak C-18 cartridge followed by chromatography on a Sephadex G-25 (F) column (1 x 95 cm) equilibrated and eluted with 0.1 M acetic acid containing 0.2% BSA (w/v). Fractions obtained from the descending portion of the single peak of radioactivity obtained, produce maximal binding and sensitivity in RIA. The LR-16 has less than a 0.01% cross-reaction with substance P and neuromedin B and has no cross-reaction with any structurally unrelated gastroenteropancreatic (GEP) peptide tested. Synthetic porcine GRP-(1-27) is used as standard for radioimmunoassay.

The buffer system used in the GRP RIA consisted of .06M Na_2HPO_4,

.05% NaN_3, .01M EDTA. The pH was adjusted to 8.0 with phosphoric acid and then .2% peptone was added to the buffer. The LR16-10 or LR-16-09 antiserum was used and they have equal affinity for GRP. Standard stocks of 10 pmol/ml of bombesin and GRP were used and serial dilutions ranging from 6.25 fmol/ml to 1600 fmol/ml were used to construct standard curves for GRP. The total incubation volume was 1.0 ml and the sample, standards, tracer and antiserum were added in 0.1 ml volumes. The reaction was started in all cases by adding the antiserum to the tubes. Following a 3-5 day incubation period at 4 C, the dextran coated charcoal method was used for separation. Buffer composition was: charcoal, .125%; dextran, .0125%. 1.0 ml of ice-cold charcoal buffer and .1ml of pig plasma were added to the incubation medium.

vi) Galanin

RIA for galanin was performed using an antiserum (LR-25 raised in a rabbit subjected to multiple intradermal injections of synthetic galanin (85 ug initial, 40 ug subsequent injections) in Freund's adjuvant; bleedings were obtained 7- 10 days postinjection. Briefiy, the LR-25 is used in a final dilution of 1:15,000 for RIA, can detect 3 fmol galanin per assay tube (10% drop from initial binding) and is C-terminally directed with residues 20-25 in the galanin molecule being important for antiserum recognition. Radiolabelled galanin is prepared and purified in a manner similar to that described for GRP above. The LR-25 does not cross-react with any structurally unrelated GEP peptide tested and, on addition of 1,000,000 fmol of peptide to assay tubes, does not cross-react with peptides such as the tachykinins, the phospate acceptor peptide and gonadoliberin, which has slight sequence homology with galanin (Tatemoto et al,1983).

The buffer system used in the galanin RIA consisted of .05M Na_2HPO_4, .05% NaN_3 and .01M EDTA. The pH was adjusted to 8.6 with 5M phosphoric acid and then .8% peptone was added to the buffer. The standard stock was 100,000 fmol/ml and serial dilutions ranging from 6.25 to 3200 fmol/ml were used to construct the standard curve for galanin. The total volume of the incubation medium was 1.0 ml. The antibody,standards,tracer and sample were added to appropriate tubes in 0.1 ml volumes. The reaction was started by adding the antiserum to the tubes. Following a 3 day incubation period at 4 C, the separation of bound and free radioactivity was carried out using a dextran coated-charcoal method; the composition of the charcoal was .10% and that of dextran was .01%; 1.0 ml of ice-cold charcoal buffer and 0.1ml of pig plasma were added to the incubation medium.

vii) VASOACTIVE INTESTINAL POLYPEPTIDE

RIA for the vasoactive intestinal polypeptide was performed using antiserum Code No. 7913 (a kind gift of Dr. J. Walsh, Centre for Ulcer Research and Education, Los Angeles, CA) the characteristics

of which have been previously described (Furness et al,1981; Reid et al,1985). This RIA system detects 1 fmol porcine VIP per assay tube (10% drop from initial binding) under conditions used in this laboratory but cross-reacts with synthetic guinea pig VIP 25 times less potently.

The buffer system used in the VIP RIA consisted of .05M NaH_2PO_4, .05% NaN_3, .01M EDTA and 0.15M NaCl. The pH was adjusted to 8.6 with 1.0M NaOH. The VIP antibody was used in a final dilution of 1:1,000,000. The standard stock was 100,000 fmol/ml and serial dilutions ranging from 6.25-3200 fmol/ml were used to construct the standard curve for galanin. The total volume of the incubation medium was 1.0 ml. The sample, antiserum, standards and tracer were added in 0.1 ml volumes to appropriate tubes. Following a 3 day incubation period at 4 C, the separation of bound and free radioactivity was carried out using a dextran coated-charcoal method; the composition of the buffer was .6% charcoal and .06% dextran. 1ml of ice-cold charcoal buffer and 0.1 ml of pig plasma were added to the incubation medium.

viii) Graphs of standard curves and log-logit transforms

Representative standard curves and their respective log-logit transforms obtained routinely in specific radioimmunoassays for various peptides are presented in figures A to E. The transformed data are linear regression lines.

E) Characterization of continuous sucrose gradient fractions obtained from tissue derived from guinea pig LMMP.

i) Preliminary experiments with gradients

In initial experiments, a variety of different continuous sucrose gradients were constructed ranging from 10% to 60% sucrose (w/v),in an attempt to establish the most suitable gradient for separating partially purified varicosities containing peptide immunoreactive entities.

ii) Preparation of continuous sucrose gradient.

Using the layering method, a discontinuous sucrose gradient, made up of 3.3 ml each of 30%, 40% and 50% sucrose (w/v), was constructed in 11.5 ml Beckman Ultra-Clear centrifuge tubes (13 x 51 mm). Each layer of sucrose was slowly added to the tubes using a 2.0 ml glass pipette. These discontinuous gradients were allowed to equilibrate at room temperature (22 C) for 3.0 h to slowly diffuse into a continuous gradient. The gradients were then cooled to the ultracentrifuge running temperature (4 C), 1.9 ml of the P_2 suspension was layered on top of the sucrose gradient and ultracentrifuged at 150,000 g (35,000 rpm) for 90 min using an SW41 Ti rotor. The three distinct bands which formed were designated F_a, F_b and F_c moving from top to bottom. The molarity of the sucrose at

these bands was obtained from control gradient tubes in which the P_2 was ommitted, with the aid of a refractometer. Using a Pasteur pipette, 1.5 ml aliquots were removed from the center of the bands, washed with ice cold Krebs buffer and centrifuged at 16,000 rpm for 20 min. Pellets F_a, F_b and F_c were resuspended in 1.6 ml Krebs (gassed with 95% O_2/5%CO_2) and an aliquot of 450 ul from each was used for determination of the occluded LDH activity. Aliquots of 1.0 ml were boiled at 98 C for 8 min and frozen till which time the specific RIA's which detect aNK, SP, GRP, VIP, ME and LE could be performed on each experiment.

iii) ANALYSIS OF THE DATA

Four separate experiments were performed and the neuronal contents from each fraction were reported as the mean values ± the standard error of the means. The ratio of neuropeptide in P_2 to that in F_b was also reported. A one tail Student's t-test for paired observations was used to compare the values obtained in the P_2 with those obtained in the other 3 gradient fractions. Significance at the $p < .05$ was accepted. Differences between the contents of neuropeptides within each fraction were analyzed by a two tail t-test for paired observations at a .05 level of significance.

F) BASAL RELEASE OF TACHYKININS

The P_2 synaptosomes were isolated using the standard protocol described above. Following resuspension of the P2 (obtained from 3 guinea pigs) in 12.5 ml of Locke's buffer, the synaptosomes were then equilibrated at 37 C for 60 min. Twelve samples (1.0ml P_2/sample) were incubated with 5mM bacitracin for 7 min and then centrifuged at 12,500 g for 90 sec using the Savant centrifuge. The supernatants (S_3) containing the TK's were quickly removed with separate pasteur pipettes and placed on ice. Six of the sample S_3 supernatants were ultracentrifuged at 105,900 g for 5 min, using a TLA 100.3 ultracentrifuge set at 2 C while tne other six remained on ice. Then, supernatants from both low speed (S_3) and high speed centrifugations (S_4) and the resuspended pellets obtained from the ultracentrifugation (P_u) were boiled for 8 min and stored at -86 C until the individual RIA's for SPLI and alpha-NKLI could be carried-out. Sample blanks and content of both immunoreactive entities were determined for the S_3, S_4 and P_u fractions.

G) DEGRADATION STUDIES

Following the resuspension of the P_2 in Krebs buffer, a period of 30 min was allowed for equilibration. Then P_2 suspensions containing 200 ug of P_2 protein were incubated with 40nM exogenous porcine SP in the presence or absence of enzyme inhibitors over a period of 60

min. The total incubation volume for each sample was 400 ul. In incubations with enzyme inhibitors present, the P_2 was preincubated for 15 min with the inhibitor(s) and then the exogenous SP was added to start the reaction.

The SPLI in the supernatants which were boiled following centrifugation at 12,500 g, was quantitated by RIA and the basal release of SPLI was subtracted from these values. This gave the true value of the exogenous SP remaining following incubation with the P_2. The concentration of exogenous SP added to the tubes at t = 0 was also measured by RIA.

H) ISOLATED GUINEA-PIG ILEUM PREPARATIONS

i) Electrically stimulated guinea-pig ileal segments
Measure contractions mediated by Acetylcholine.

Adult male or female guinea-pigs weighing 300-400 g were sacrificed by a blow on the head and subsequent exsanguination. The intestine was exposed by a midline incision and the entire ileum, with the exception of the terminal 10 cm, was removed. Ileal segments (1.5-2.5 cm in length) were dissected free of mesenteric attachments and cleaned of luminal contents by carefully passing Krebs' solution through them. Segments were suspended in 10 ml organ baths containing Krebs' solution (pH 7.4) maintained at 37°C and bubbled with 95% 02/5% C02. The composition of the Krebs' solution (millimolar) was as follows: KCl,4.75; CaCl2,2.54; MgS04,1.19; KH2P04,1.19; NaCl,118; NaHC03,25; Dextrose,11.0.

Electrical field stimulation of the preparations was achieved by placing a stainless-steel needle at the bottom and another at the top of the ileal segment. A load of 1.0 g was applied to each preparation and responses were obtained isotonically using Harvard Apparatus rotary motion transducers (Harvard Apparatus Co., Inc., Millis, MA) and a Rikadenki ink-writing recorder (Rikadenki Kogyo Co., Ltd., Tokyo, Japan). Trains of supramaximal rectangular pulses of 1 msec duration were delivered from a Grass model S-5D stimulator (Grass Instruments Co., Quincy, MA) at 0.2 Hz continuously for the duration of each experiment (3-4h). Preparations were allowed an equilibration period of at least 1h, during which they were washed every 10 mins, before exposure to any drugs.
In experiments in which adenosine receptor antagonists were also included, the antagonist was equilibrated with each tissue for 30 min without stimulation and for a further 30 - 45 min with stimulation.

ii) Atropinized longitutinal muscle-myenteric plexus preparation (LMMP).

Adult male or female guinea pigs of either sex weighing 350-400 g were sacrificed by a blow on the head and subsequent exsanguination.

The intestine was exposed by a midline incision and the entire ileum was removed except for the terminal 10 cm. Ileal segments (10 cm in length) were threaded onto 1.0 ml glass pipettes and the LMMP was stripped-off with non-absorbent cotton wool. It was then folded twice to 1/4 its length, secured with surgical silk and placed in a 10 ml organ bath containing Krebs solution (pH 7.4) maintained at 37 C and gassed with 95% O_2 and 5% CO_2. Preparations were allowed an equilibration period of 1.0 hour, during which they were washed every 10 min, before exposure to any drugs.

Approaches used to release tachykinins from myenteric nerve endings:

a) Electrically-stimulated contractions of the atropinized LMMP

Electrical field stimulation was achieved by securing the bottom of the LMMP onto a stainless-steel hook and the top to a flexible stainless steel wire. A load of 0.5 g was applied to the preparation and recordings were made isotonically using Harvard Apparatus rotary motion transducers and a Rikadenki ink writing recorder as described earlier. Trains of supramaximal rectangular pulses of 0.75 msec duration were delivered with an Anapulse Stimulator (W-P Instruments, New Haven, Conn. USA) at a frequency of 20 Hz intermittently for periods of 25 seconds. The LMMP was stimulated every 15 minutes. Following each stimulation in the presence of the nucleoside, the control response to ES was reestablished prior to testing the effect of another dose. Acceptable control stimulations varied by less than 10% of each other. The nucleoside was included in the bathing fluid during the last 3 min of the 15 min cycle.

b) Cholecystokinin octapeptide (CCK8) mediated contractions of the atropinized LMMP

A concentration of 5nM CCK8, which is equiffective with the tachykinergic response to ES, was used to elicit contractions of the atropinized LMMP. The stimulation cycle used for CCK8 responses was as above. In those experiments in which adenosine receptor antagonists were included in the bathing fluid, the antagonists were allowed a 60 min equilibration with the tissue prior to its exposure to any agonists.

iii) Incubation with the SP-A and Tetrotoxin.

Contractile responses of the ileum to ES, CCK8, SP, KCl, Histamine or GRP were first obtained in the absence of the SP-A and then following prexposure of the tissue to $4x10^{-5}$M SP-A for 10 min without washing it out. Each tissue was exposed to only one agonist and one dose of the antagonist in order to eliminate the possibility of residual effects of previous additions of both agonists and antagonists. A similar protocol was used for determining the TTX-sensitivity of the responses to the various agonists.

iv) Responses to Gastrin Releasing Polypeptide

The dose response relationship of the effect of GRP on the unstimulated LMMP was obtained non-cummulatively. Each dose was tested on a different tissue because of severe tachyphylaxis. The effect obtained in either the presence of TTX or the SP-A was compared to control responses to GRP in different tissues. The data was analysed using a t-test for unpaired samples.

v) Analysis of the effects of nucleosides on guinea pig ileal contractions

The dose-inhibition curves obtained for nucleoside inhibition of ileal contractions (both LMMP and intact ileal segments) were analysed using the nonlinear curve fitting technique described by DeLean et al. (1978). This was achieved by using the ALLFIT program available from the Biomedical Computing Technology Information Center (BCTIC) of Vanderbilt Medical Center (Nashville, TN). The EC_{50} values for the nucleoside analogs were obtained from the fitted curves. Standard error of the means (S.E.M.) were also calculated for all the data points on the curves.

vi) Determination of the affinity of xanthine analogs for adenosine receptors present at the guinea pig ileum

Dose-effect curves for nucleosides in the presence of incrementing concentrations of adenosine receptor antagonists were similarly obtained using the ALLFIT program. The EC_{50} values were obtained from the fitted curves. The shifted EC_{50}'s were compared with controls obtained in the absence of antagonist using the same preparations for cholinergic responses, and different preparations for tachykinergic responses since a relatively lengthy drug addition cycle was required. These values were used to calculate the dose ratios (DR) at each concentration of the antagonist. These data were used to construct Schild plots according to Arunlakshana and Schild (1959).
The isoboles were presented as the least squares regression lines and the 95% confidence intervals of the slopes of the lines were calculated according to Goldstein (1964). If the intervals included the slope of 1.0 then the slope of the isobole was not considered significantly different from unity. The intercept of the isobole with the x-axis indicated the pA_2 value (affinity of the antagonist for the receptor) of the antagonist.

Representative standard curves for the various peptides used. The left panel of each figure (A-E) shows the B/F ratios plotted against the concentration of the standard peptide used. The right panel of each figure shows the log-logit transform of each standard curv . Calculation of respective peptide immunoreactivities were made using the transformed curves.

Figure A

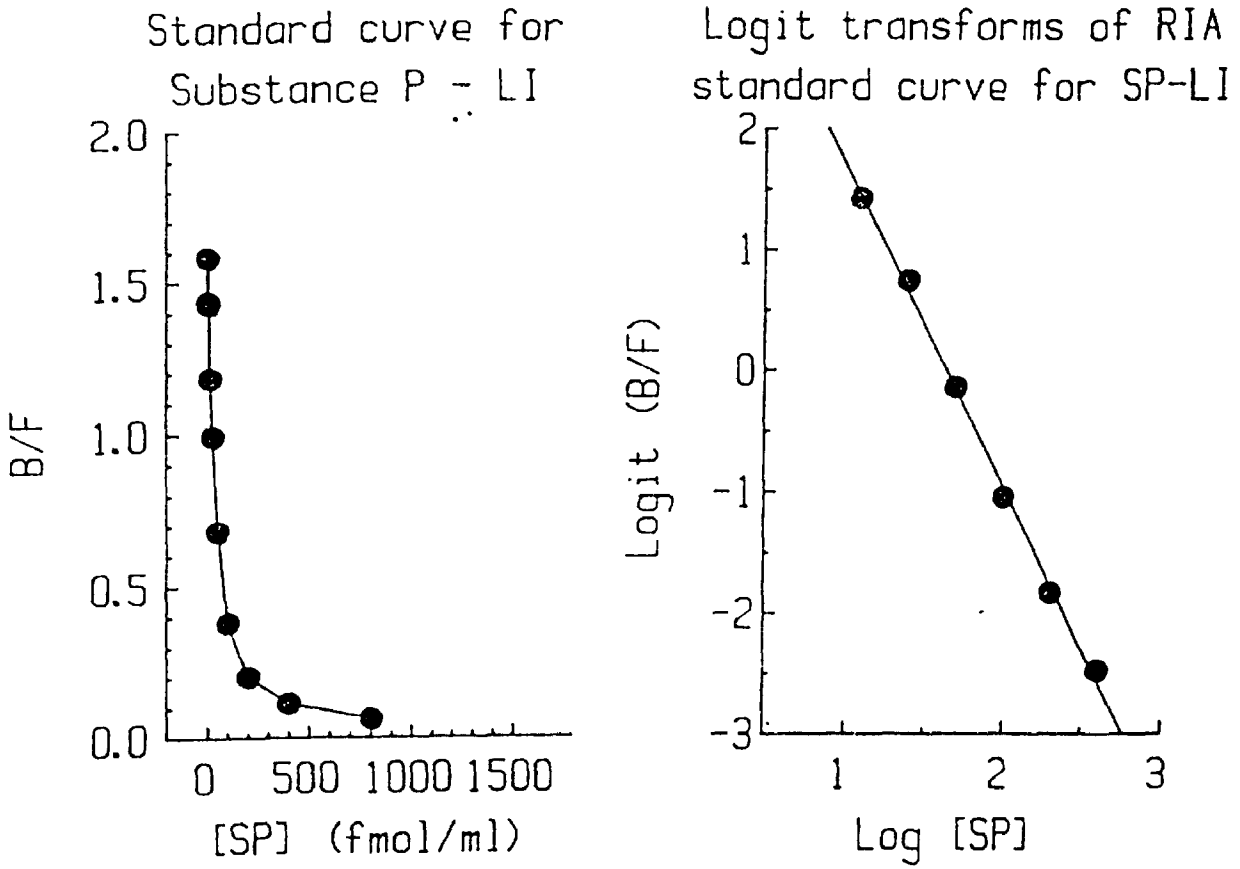

Standard curve for
Substance P - LI
B/F
2.0
1.5
1.0
0.5
0.0
0
500
1000
1500
[SP] (fmol/ml)
Logit transforms of RIA
standard curve for SP-LI
Logit (B/F)
2
1
0
-1
-2
-3
1
2
3
Log [SP]

Figure B

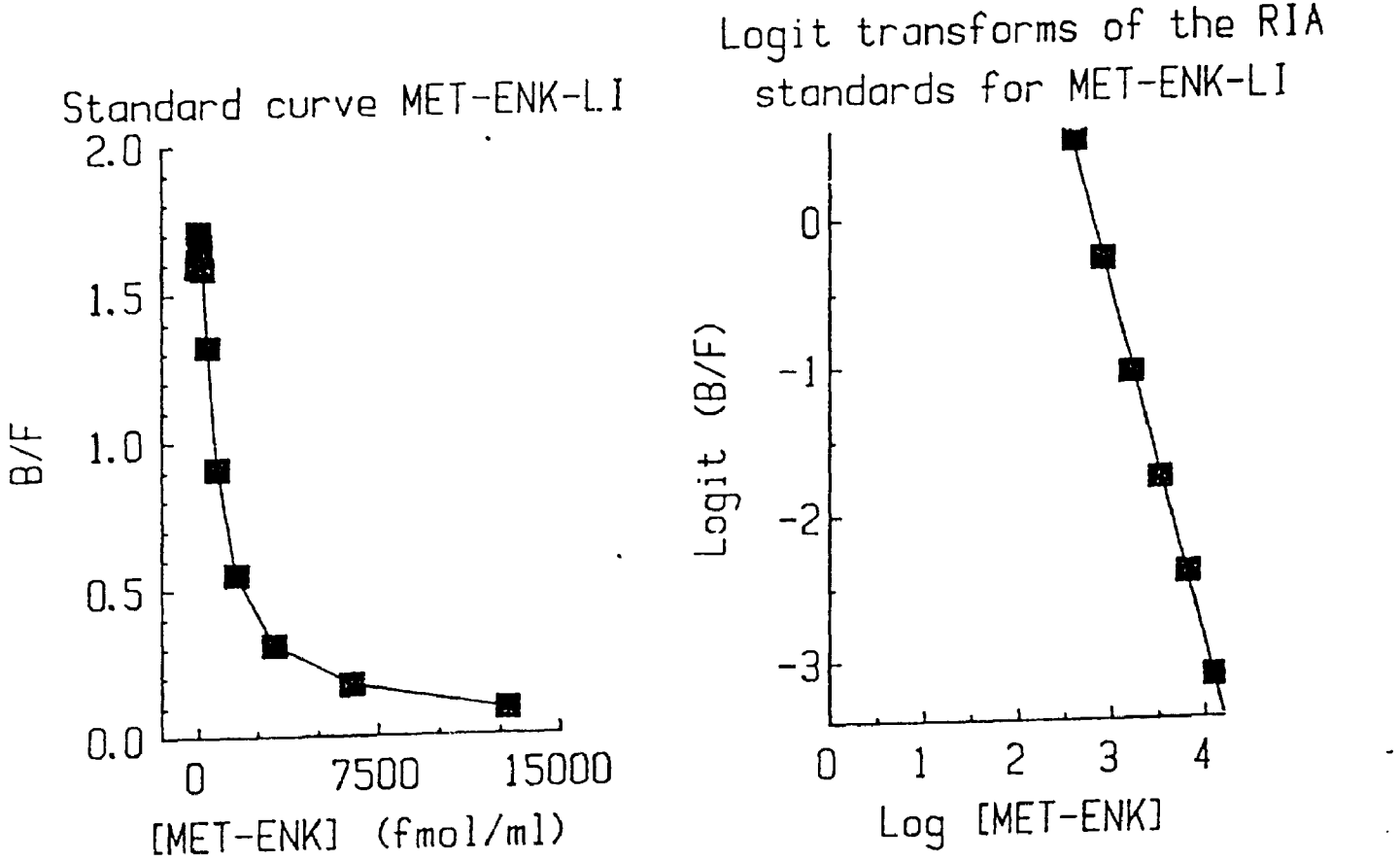
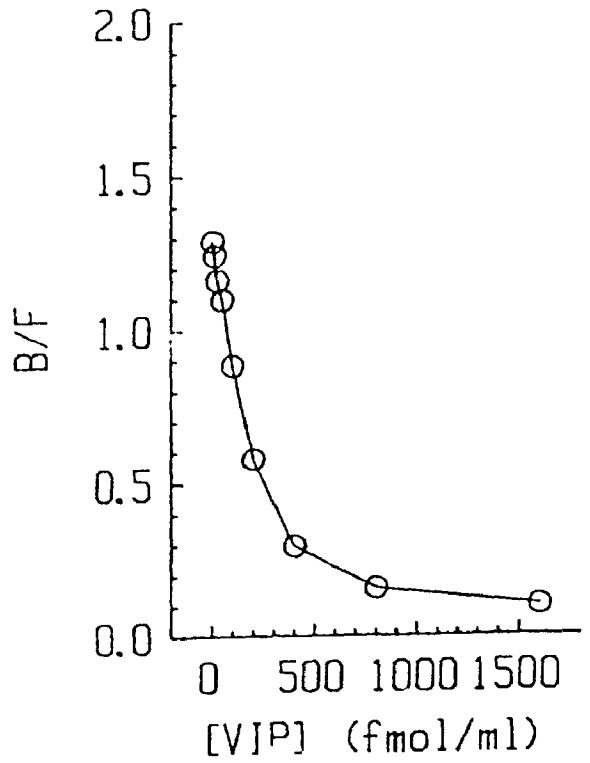

Standard curve MET-ENK-LI
2.0
1.5
1.0
0.5
0.0
B/F
0
7500
15000
[MET-ENK] (fmol/ml)
Logit transforms of the RIA standards for MET-ENK-LI
0
-1
-2
-3
Logit (B/F)
0
1
2
3
4
Log [MET-ENK]

Figure C

Standard curve for VIP-LI



Logit transforms of RIA standard curve for VIP-LI

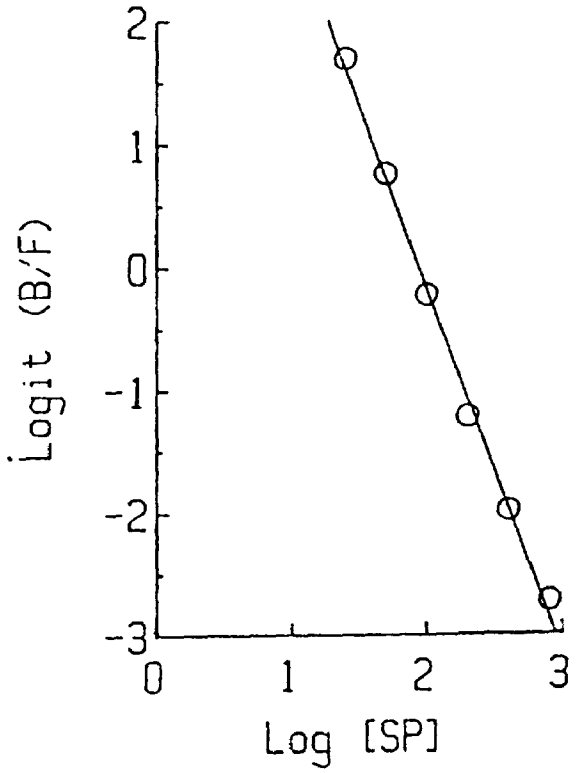
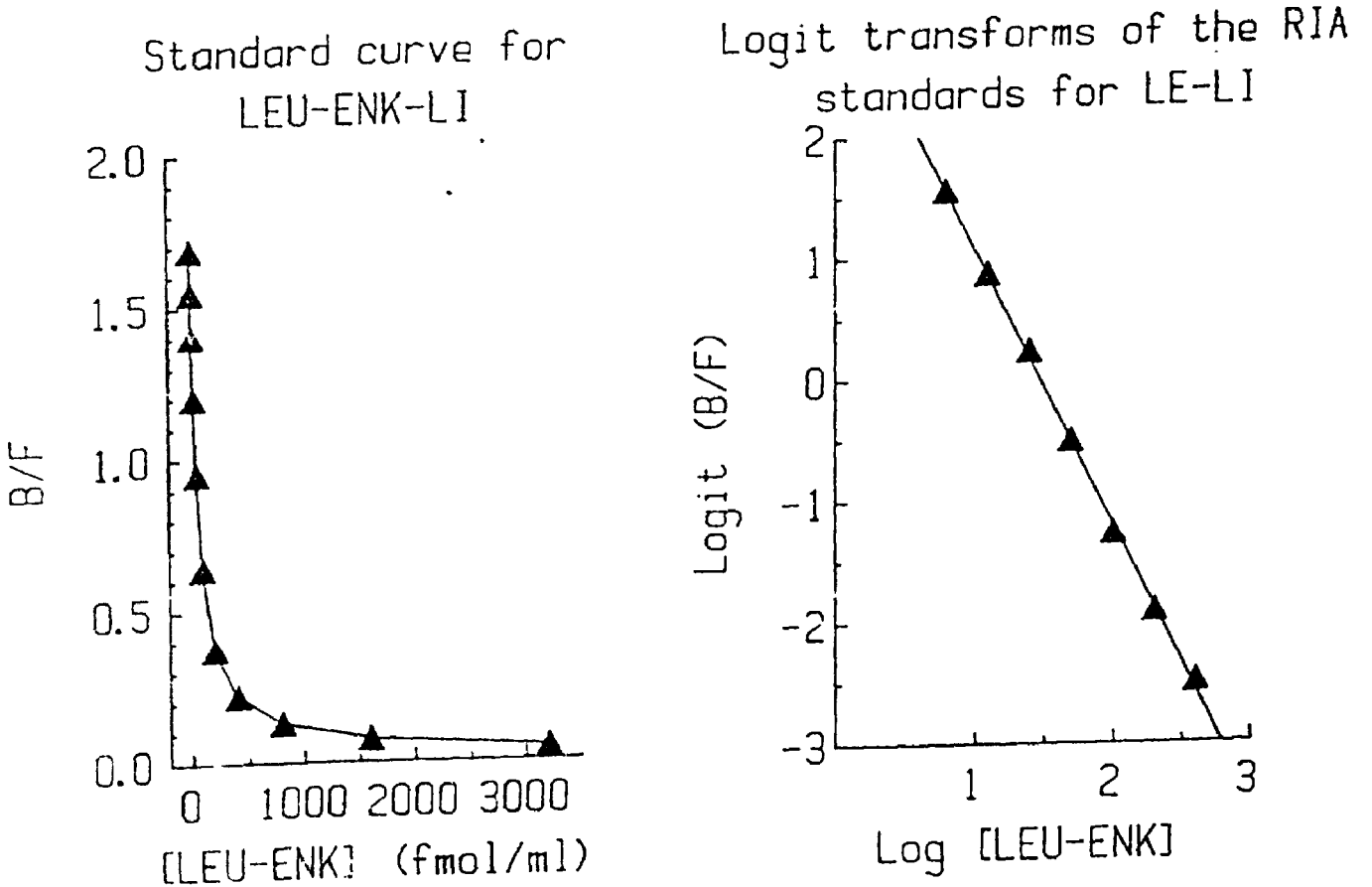


Figure D


Standard curve for
LEU-ENK-LI
B/F
2.0
1.5
1.0
0.5
0.0
0
1000
2000
3000
[LEU-ENK] (fmol/ml)
Logit transforms of the RIA
standards for LE-LI
Logit (B/F)
2
1
0
-1
-2
-3
1
2
3
Log [LEU-ENK]

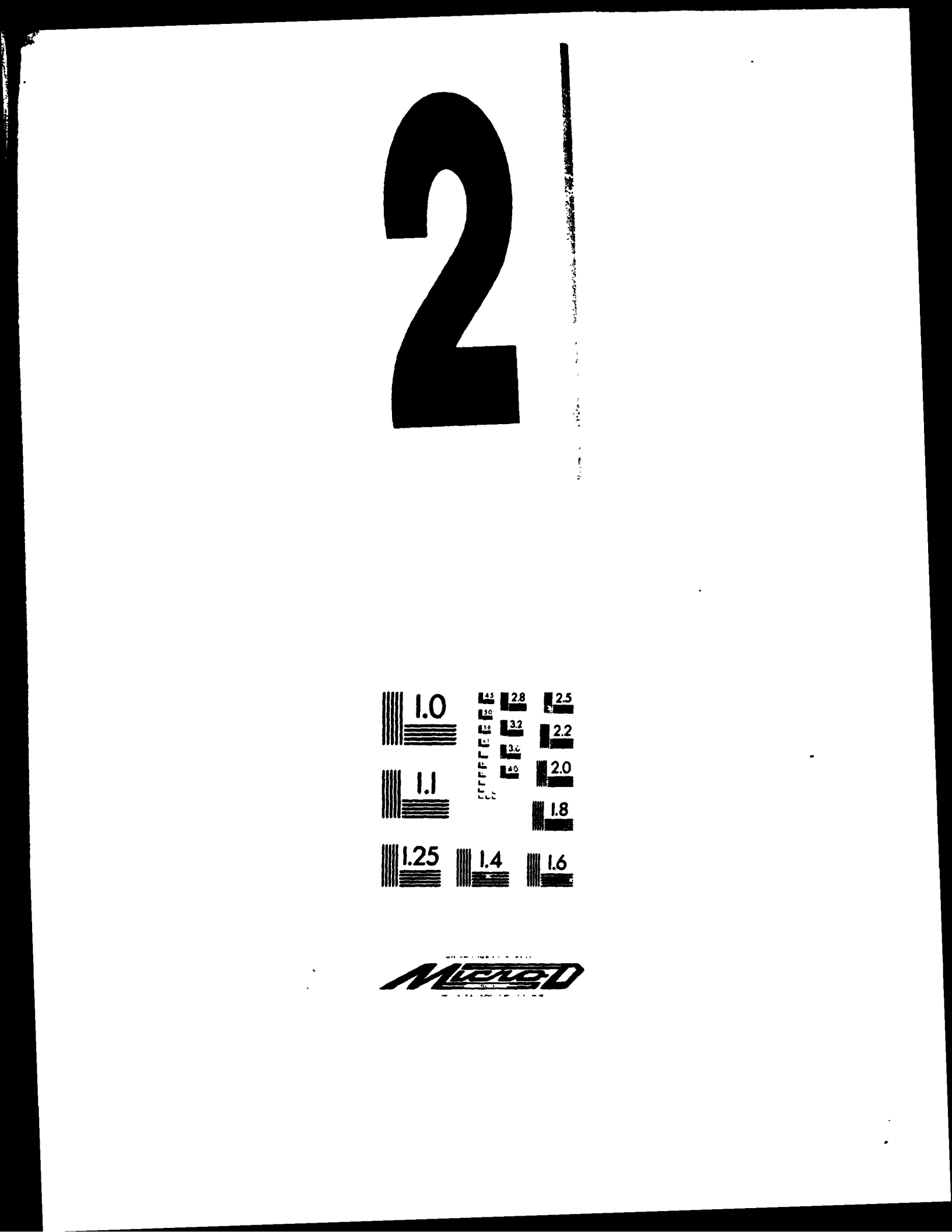
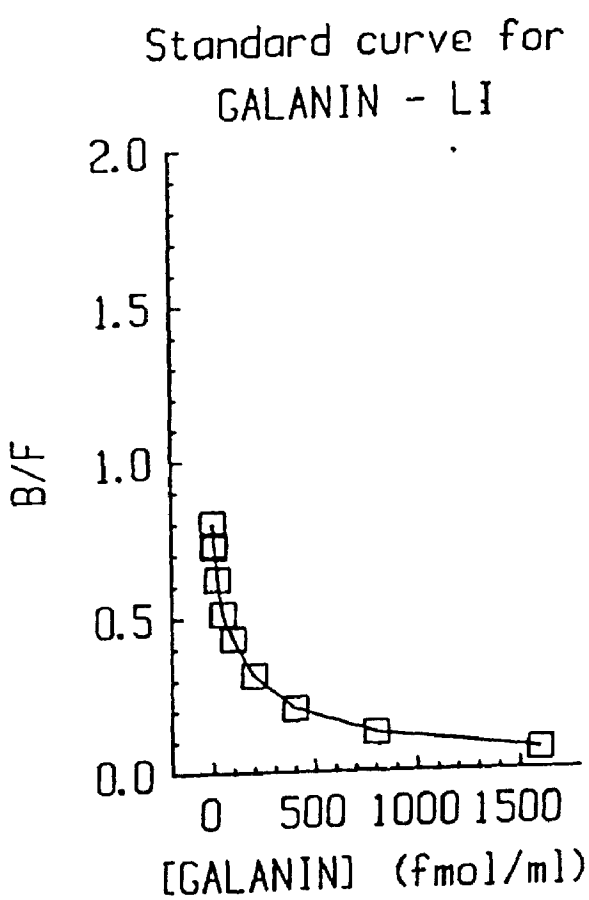
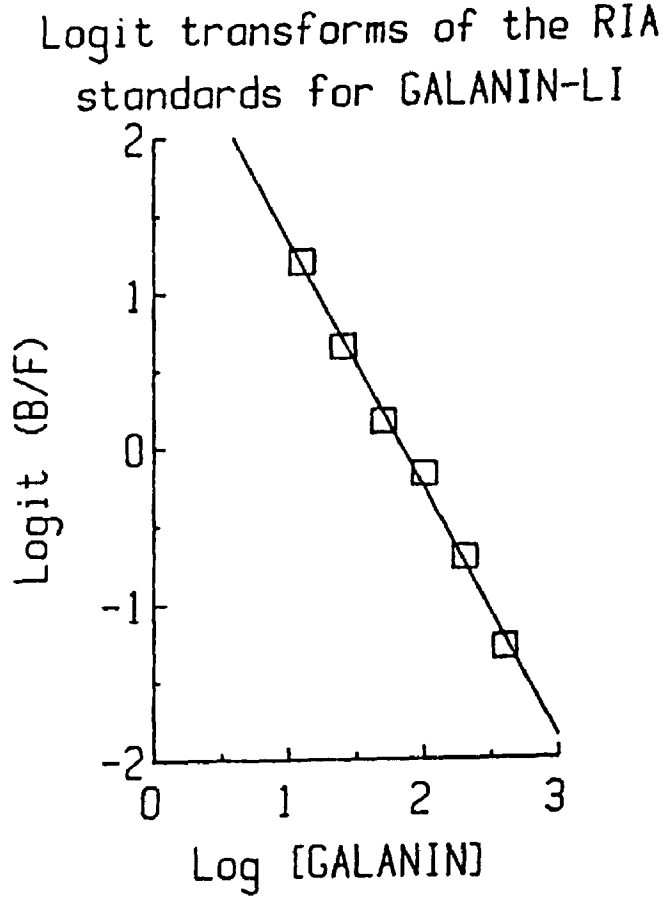

2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
2.5
2.2
2.0
1.8

Figure E


Standard curve for
GALANIN - LI
2.0
1.5
1.0
0.5
0.0
B/F
0
500
1000
1500
[GALANIN] (fmol/ml)


Logit transforms of the RIA
standards for GALANIN-LI
2
1
0
-1
-2
Logit (B/F)
0
1
2
3
Log [GALANIN]

RESULTS.

Electrically stimulated guinea pig ileal segments (0.2Hz,1msec) were used to determine the biological activity of the nucleosides. The stable adenosine analogs CHA, R-PIA, CPA, MCPA and NECA inhibited the twitch response of the stimulated ileum preparation in a dose-dependent manner. The Log dose-effect curves are shown in figure 1. The EC_{50} value obtained for NECA is not significantly different from those obtained for the other analogs ($p > .05$). The EC_{50} values are shown in table 1.

In the presence of NBMPR (6.25×10^{-7}), the dose-effect curve to adenosine was significantly shifted ($p<.05$) to the left by an order of magnitude (EC_{50} in the absence of NBMPR was $1.1 \times 10^{-6}M \pm 9\%$)and in the presence of NBMPR was $1.5 \times 10^{-7}M \pm 8\%$; n=3). The uptake inhibitor had no apparent effect on the dose-effect curves to R-PIA, CHA or NECA.

Biological activities of several R and S- diastereoisomers of N^6-substituted analogs of adenosine.

Both R- and S- diastereoisomers of N^6-substituted analogs of adenosine inhibited the twitch response of the stimulated ileum preparation in a dose-dependent fashion. The results are presented in fig 2a to 2c. Within each pair, the R-isomer was found to be more potent than the S-isomer. The largest difference in potency was obtained with PBA, whereby the R-isomer was approximately 40 times

more potent than the S-isomer. R-PIA was about 10 times more potent than S-PIA.

Antagonism of inhibitory effects of nucleosides at the stimulated ileum by xanthine analogs.

Dose-effect curves to several nucleosides were obtained in the absence and presence of incrementing concentrations of theophylline or DPSPX. Schild plots of these data are shown for theophylline in figure 3a to 3d. Linear isoboles with unit slopes ($p>.05$) were obtained for the analogs NECA, R-PIA, CHA and CPA against theophylline and the pA_2 values obtained are listed in table 1.

Schild analysis using DPSPX (see fig 4) showed linear isoboles with unit slope ($p>.05$) for both CHA and MCPA while NECA displayed a curvilinear isobole. The 95% confidence intervals calculated for the dose-ratios showed that, at a DPSPX concentration of 5 uM the values obtained for NECA were significantly different ($p<.05$) from those obtained for either of the A_1 agonists. Further, the values for NECA and CHA at 2 uM were also significantly different ($p<0.05$). Similar analysis of the isoboles at lower antagonist concentrations showed no such significant differences. The deviation of the Schild isobole from unit slope thus commences at an ordinate value (DR-1) approaching 1.0. For comparison purposes, the linear isoboles for CHA and NECA obtained against theophylline are also presented in fig 4. The pA_2 values obtained for several adenosine analogs against DPSPX are shown in Table 1.

Contractile responses of the atropinized LMMP.

Tracings showing representative contractions of the atropinized guinea pig LMMP to 5nM cholecystokinin octapeptide and 5nM Substance P are presented in fig 5a. These agonists produced responses with a rapid onset achieving a maximum in less than 30 seconds. In the presence of .04mM SP antagonist [d-pro^{4},d-trp^{7,9,10}] SP(4-11) (SPA) these contractions were abolished. Representative responses to electrical stimulation (20Hz,.75msec) shown in fig 5b, were rapid in onset achieving a maximum in 15 seconds. These responses were dose-dependently inhibited by the SP-A. A dose of 2uM SPA produced no apparent inhibition, 20uM SPA resulted in about 50% inhibition and .04mM SPA inhibited about 70% of the response. Similar findings were obtained in two other experiments.

Desensitization of the atropinized LMMP with Substance P.

In the presence of 2uM atropine, 1.0 uM ACh failed to contract the guinea pig ileum LMMP, a dose that produces maximum contraction in the absence of atropine. Fig 6 shows tracings from a typical desensitization experiment of the LMMP to 0.5uM SP. The response to ES reached a plateau rapidly within 15 seconds and then declined to a second sustained plateau in the following 30 seconds. After 3 successive 15min incubation-wash periods of the atropinized LMMP with 0.5uM SP, ES failed to produce any contraction, whereas 50uM histamine produced a control contraction of the tissue. Five minutes following a wash, a small response to ES was evident. Ten minutes

after the wash the control response to ES was re-established. In the presence of 5.0uM TTX the response to ES was abolished.

Antagonism by [d-Pro^{4},d-Trp^{7,9,10}] SP(4-11) and TTX at atropinized LMMP.

Contractile responses of the atropinized LMMP were determined in the absence or presence of either TTX or the SPA. The findings are shown in table 2. In the presence of 5uM TTX, responses to 5nM CCK8 and ES were abolished. Responses to the depolarizing stimulus K^{+} (10mM) or 5nM SP were not affected by TTX and those to 50uM histamine were significantly inhibited but not abolished. The SPA (40uM) completely blocked responses to both CCK8 and 5nM SP whereas only 65% of the contractile response to ES was blocked. The response to 10mM KCl was not significantly antagonized. Histamine responses (50uM) were significantly diminished but not abolished in the presence of the SPA.

Inhibition of contractile responses to either CCK8 or ES by adenosine analogs.

The stable nucleoside analogs CPA, NECA and 2PAA dose dependently inhibited contractions of the LMMP to 5nM CCK8 as shown in fig 7a. All analogs achieved 100% inhibition. The field stimulated responses illustrated in fig 7b were dose-dependently inhibited by CPA and NECA, achieving only a 65% maximum inhibition. Table 3 shows both EC_{50} values obtained with CCK8 responses and maximum inhibition obtained with ES responses. The rank order of potencies for

inhibition of CCK8-mediated responses was CPA > NECA > 2PAA (differences between agonists different at .05 level). The rank order of potencies for inhibition of ES-responses was CPA = NECA ($p > .05$).

Antagonism of inhibitory effects of nucleosides at the atropinized LMMP by xanthine analogs.

Dose-effect curves to nucleosides were obtained in the absence and presence of incrementing concentrations of either DPSPX or CPDPX. Schild plots of these data are presented for CPDPX in fig 6a and for DPSPX in figs 8b and 8c. A linear isobole with a unit slope was obtained for NECA against CPDPX and the pA_2 value was 8.0. Analysis with DPSPX gave linear isoboles with unit slopes for both NECA and CPA and their respective pA_2 values were 6.52 and 7.0. These values were significantly different from the pA_2 value obtained against CPDPX ($p < .05$).

Preparation of purified myenteric varicosities.

Purified myenteric varicosities were obtained with an average yield of 178 $\pm$ 26 ug of protein per guinea pig (n=11). Assays for occluded LDH, as a measure of cytoplasm enclosed by a membrane, were routinely performed on each PV preparation and yielded a mean of 1312 $\pm$ 85 LDH units/mg of protein (n=11). A comparison of the yields per animal and occluded LDH values is shown in table 4. Comparison of the fraction of total binding of the ligands which was displaceable (i.e.,specific binding) with the occluded LDH unitage

per milligram of protein revealed the expected positive relationship, which is shown in figure 9a. As a consequence, preparations yielding less than 700 U/mg of occluded LDH were discarded.

Determinations of occluded LDH activity were also obtained from a single preparation of PV at several intervals over a 2.0 h period after its preparation. No significant decline in occluded LDH unitage was detected.

Electron microscopy of the purified varicosity fraction revealed that the preparation was enriched with varicosites with intact resealed plasma membranes. These varicosities varied in size from 0.5 to 1.5 um in diameter and contained vesicles of various types, mitochondria and occasionally glycogen granules (fig 9b). Free mitochondria were only rarely seen in the PV fraction while other membranous profiles, some of which had resealed, were also present. The vesicles present in the varicosities could be categorized according to the criteria of Furness and Costa (1980).

Effect of temperature on binding of [^{3}H]R-PIA to PV

Specific binding of labeled R-PIA to PV was determined at various incubation times at 0, 22 and 37 degrees C. Equilibrium was achieved after 10 min of incubation at all three temperatures. The lowest amount of specific binding was evident at 0 C, an intermediate amount at 22 C and the highest amount at 37 C. All subsequent

binding experiments were performed at 22 C with an incubation time of 12 min.

Effect of protein concentration on specific binding of [^{3}H]R-PIA to PV. The total and nonspecific binding of labeled R-PIA at a concentration of 20nM was determined for several concentrations of the PV fraction ranging from 50 to 200 ug/assay tube. The maximum specific binding of the ligand comprised 40 % of the total binding and was achieved at protein concentrations greater than 80 ug/tube. Similar determination using [^{3}H]NECA yielded 60% specific binding. (Similar results were obtained in 3 separate experiments for each radioligand).

In all subsequent binding experiments the PV protein concentration used in each assay tube was 80 to 100 ug.

Competition with [^{3}H]R-PIA and [^{3}H]NECA by various unlabeled nucleosides for specific binding at PV.

The specific binding of the radioligands to binding sites at PV achieved 37.2 $\pm$ 1.6% (n=5) of total binding for [^{3}H]R-PIA and 64 $\pm$ 2.2% (n=6) for [^{3}H]NECA. Specific binding was determined in the presence of 100 uM unlabeled R-PIA or NECA respectively.

Competition curves for R-PIA, NECA, 2-chloroadenosine, adenosine-N^1-oxide, purine riboside and inosine as displacers of [^{3}H]R-PIA and [^{3}H]NECA were established. The curves are shown in figures 10a and 10b. The concentrations of labeled ligand used were 5nM for R-PIA

and 20nM for NECA.

Displacement of 100% of specifically bound radioligand was achieved by 2-chloroadenosine against both labeled nucleosides and by adenosine-N^1-oxide against R-PIA. Adenosine-N^1-oxide, purine riboside and inosine achieved 60, 70 and 74% displacement, respectively, against [^{3}H]NECA. Purine riboside and inosine achieved 76 and 64% displacement, respectively against [^{3}H]-R-PIA. The iteratively fitted curves for each competitor were used to obtain IC_{50} values which are shown in table 5.

Competition curves with [^{3}H]R-PIA and [^{3}H]NECA (20nM) against theophylline were established and are presented in figure 11. Displacement of approximately 80% of the specifically bound radioligand was obtained for both ligands at the highest concentration of theophylline used. The IC_{50} values obtained from the iteratively fitted curves were 2.3×10^{-4}M and 1.3×10^{-4}M against labeled R-PIA and NECA, respectively. No significant difference exists between these IC_{50} values ($p > .05$). Determination of the competition by theophylline of [^{3}H]R-PIA at 5×10^{-9}M yielded the expected lower IC_{50} value of 9.0×10^{-6}M.

<u>Comparison of activity of nucleosides as displacers of radioligand binding with their efficacies as inhibitors of acetylcholine release at the stimulated ileum preparation (.2Hz, 1msec).</u>

Figures 12a and 12b illustrate correlations between the IC_{50} values

obtained for nucleoside analogs as displacers of the specific binding of labeled NECA (figure 12a) and labeled R-PIA to PV, and the EC_{50} values for the same compounds as inhibitors of acetylcholine release at the stimulated ileum preparation. The slope of the regression line through the data points for [3H]NECA binding was 0.893 ± 0.182 with a correlation coefficient of 0.943. For [3H]R-PIA the slope of the regression line was 1.017 ± .042 with a correlation coefficient of 0.998. The slopes of the lines were not significantly different from unity ($p>.05$).

Displacement of [^{3}H]-nucleoside analogs from PV by selective adenosine receptor analogs.

Displacement curves for 20nM [^{3}H]- CHA, R-PIA and NECA were determined on PV using their unlabelled counterparts as displacers. The maximum specific binding for these radioligands expressed as a percentage of the total binding achieved by each radioligand, was 41%, 37% and 64% respectively. The displacement curves are shown in figure 13. The IC_{50} values, derived from iteratively fitted curves, were not significantly different from each other ($p>.05$).

Displacement of ^{3}H-CHA binding by unlabelled NECA generated the curve shown in figure 14a. Comparison with the curve for unlabelled CHA is also shown. Similarly, displacement of ^{3}H-NECA by unlabelled CHA and R-PIA generated the curves shown in figure 14b. Comparison with the curve for unlabelled NECA is also presented. In those cases where 100% displacement of the specific binding was achievable by

the unlabeled compounds, the IC_{50} values, derived from iteratively fitted curves, are reported in table 6.

Displacement of nucleoside radioligands from PV by the A_1 adenosine receptor antagonist DPSPX.

Displacement curves for [^{3}H]- CHA and NECA were determined on PV using unlabelled DPSPX as the competitor and a representative experiment is illustrated in figure 15. The IC_{50} data is given in table 6.

SATURATION BINDING ISOTHERMS FOR ^{3}HNECA AND ^{3}HCHA ON PV.

Saturation isotherms were obtained for ligand concentrations ranging from 0.25-100nM. Specific binding of [^{3}H]NECA and [^{3}H]CHA to guinea pig PV was saturable (see figures 16a and 16b respectively). Specific binding at a ligand concentration of 10nM represented greater than 90% for NECA and less than 50% for CHA. For Scatchard analysis, saturation isotherms were analysed using the EBDA scatchard fitting program. From initial analysis it was calculated that the data is best fitted by a one site model. For both radioligands, the slopes obtained assuming a two site model were not significantly different from each other ($p>.05$). Scatchard plots fitted with a one site model showed the presence of high-affinity binding sites at PV for [^{3}H]NECA (see figure 17a) and [^{3}H]CHA (see figure 17b) with K_d and Bmax values reported in tables 7 and 8 respectively.

Stereospecificity of binding of labeled NECA and R-PIA to PV.

N^6-(R)-1-phenyl-2-butyladenosine and N^6-(S)-1-phenyl-2-butyladenosine displaced the specific binding of labeled NECA (20nM) or R-PIA (10nM) with similar potencies (IC_{50} = $2X10^{-8}M$ ± 15% against R-PIA; maximum displacement of ^{3}HNECA binding = approx. 50%). R-PIA and S-PIA also displaced the specific binding of each of these radioligands with similar potencies. Three separate experiments were carried-out using concentrations ranging from $1X10^{-9}M$ - $1X10^{-4}M$.

Effect of boiling PV on specific binding of 20nM [^{3}H]-R-PIA.

The total binding of 20nM R-PIA to intact PV (100 ug/tube) was 3.797 ± .02 pmoles/mg and the nonspecific binding (determined in the presence of 100uM R-PIA) was 2.811 ± .03 pmoles/mg. Following the boiling of PV aliquots for 5 min. at 97 C, the total binding was 4.561 ± .14 Pmoles/mg and the nonspecific binding was 3.845 ± .16 Pmoles/mg. These represent the pooled data from 3 separate PV preparations.

Specific binding of [^{3}H]NECA to various forms of the PV fraction.

The specific binding of [^{3}H]NECA to intact PV, broken membranes from PV and boiled membranes from PV was determined. Nonspecific binding was estimated in the presence of 50 uM NECA. The results from 4 such experiments are presented in table 9. The specific binding obtained at PV is the same as that obtained at the broken membrane preparation ($p>.05$) and the total binding is significantly enhanced at the Br. membranes ($p<.05$). With boiling, the specific binding of

^{3}HNECA to the broken membrane preparation drops to zero and the total binding equals the amount of nonspecific binding obtained at PV ($p>.05$).

Saturation binding isotherms for [^{3}H]NECA and [^{3}H]CHA on the mitochondrial fraction (MITF).

Saturation isotherms for radioligands were constructed over ligand concentrations from 0.25-100nM. Specific binding obtained for both [^{3}H]CHA and [^{3}H]NECA at the MITF was saturable and the saturation isotherms are shown in figures 18a and 18b respectively. Specific binding for these radioligands at the MITF constituted greater than 90% and less than 20% of the total binding for labeled NECA and CHA respectively. Scatchard plots (EBDA analysis) were best fitted by a one site model (difference in slopes for two sites, $p>.05$) and revealed the presence of high-affinity binding sites for both radioligands. The calculated K_d and Bmax values are reported in tables 7 and 8 and representitive Scatchard plots are shown in figures 19a and 19b.

Characterization of peptide-immunoreactivities in isolated myenteric varicosities derived from guinea pig longitutinal muscle-myenteric plexus strips.

Gastrin-like and PYY-like immunoreactivity were not detectable in the PV preparations. Figures 20 to 23 illustrate mean dose-inhibition relations for SPLI, GALLI, VIPLI and GRPLI respectively. SPLI, VIPLI and GRPLI in the PV preparations produced dose-

inhibition curves close to parallel or parallel with those produced by their respective synthetic peptides. Although the GALLI content was appreciably lower than that of other peptides, a dose-inhibition relation similar to that of porcine galanin was found at the top portion of the standard curve. The dose-inhibition curve of LELI closely paralleled that of synthetic LE while that of MELI was close to but not completely parallel to that of synthetic ME (see figures 24 and 25 respectively).

Table 10 lists the amounts of neuropeptides measured by RIA in 7 PV preparations. SPLI was found to be the predominant immunoreactive entity present and MELI was present in greater concentrations than the structurally related LELI. GALLI and GRPLI's were present in relatively low concentrations in the PV.

Except for VIPLI, acidification of the heat-inactivated PV preparations did not significantly increase (in 4 cases a decrease occurred) extractable amounts of the neuropeptides. Acidification of 3 PV preparations resulted in recoveries of immunoreactivities compared to control (non-acidified) preparations: SPLI, -17.0 $\pm$ 16.4%; VIPLI, +130.4 $\pm$ 17.0%; GALLI, -35.8 $\pm$ 10.7%; GRPLI, +25.9 $\pm$ 13.4%; MELI, -10.8 $\pm$ 0.4%; LELI, -8.7 $\pm$ 4.0%.

<u>Concentration of peptide-immunoreactivities present in various fractions obtained at various stages during the isolation of the PV.</u>

Yield, occluded LDH activity and neuropeptide contents of GRPLI,

VIPLI, MELI and LELI were measured in the P_2, Fa, Fb and Fc fractions obtained at various steps in the isolation of PV from guinea pig ileal longitutinal muscle-myenteric plexus strips. The results are presented in table 11. A histogram of the occluded LDH activity measured in various fractions is plotted in figure 27. The relative amounts of peptide-immunoreactivities in the various fractions are also presented as histograms in figures 28a to 28d.

The concentration of each peptide immunoreactivity per fraction in order was: GRPLI, P_2 > Fb > Fc = Fa; MELI, P_2 > Fb = Fa > Fc; VIPLI, P_2 > Fb > Fc = Fa; LELI, P_2 > Fb > Fa > Fc; the corresponding occluded LDH activity / fraction was: P_2 > Fb > Fc > Fa. The occluded LDH activity and the neuropeptide content are highest in the P_2 fraction. The ratio of P_2:Fb peptide contents are similar for GRPLI, VIPLI and LELI. The ratio for MELI was 4-5 times greater than that for the other neuropeptides (significant difference at $p<.05$). The ratios of P_2:Fb peptide contents are presented in figure 29.

Figure 30 is a photograph taken from a typical continuous gradient obtained in these studies, illustrating clear bands at Fa (.648$\pm$ .11M), Fb (1.027$\pm$ .07M) and Fc (1.257 $\pm$ .06) sucrose density levels.

<u>HPLC characterization of peptide-immunoreactivities present in the PV</u>.

On reverse-phase HPLC using 'steep' gradient elution conditions, the PV preparations contained a number of SPLI peaks with the

predominant immunoreactive entity eluting at the retention time of synthetic SP and a smaller SPLI peak at the retention time of oxidized SP (figure 31). A smaller but readily detectable peak of immunoreactivity occurred at the retention time of alpha-neurokinin. A major SPLI peak eluted at a retention time greater than that of other tachykinins or of any C-terminal fragment of substance P. Figure 31b displays a representative 'shallow' gradient elution profile of these synaptosomal SPLI entities. The main SPLI peak eluted close to the retention time of synthetic substance P and a smaller SPLI peak eluted at the position of oxidized substance P. A small but easily detectable SPLI peak occurred at the retention time of alpha-neurokinin (figure 31b) similar to results obtained with the 'steep' gradient elution conditions (figure 31a). An SPLI peak at the retention time of beta-neurokinin was not consistently detected. Certain of its oxidized forms elute at retention times similar to that of the major SPLI peak, rendering the identification of such entities impossible. Under 'shallow' gradient elution conditions, the more avidly retained and unidentified SPLI peak was not eluted.

Enteric synaptosomal LELI eluted at an identical retention time to that of synthetic LE with a smaller peak of immunoreactivity occurring at the retention time of synthetic ME, consistent with the known cross-reactivity of this antiserum (figure 32a). The enkephalin antiserum, which recognizes both enkephalins, identified

peaks of immunoreactivity at identical retention times ot those of synthetic Leu- and Met-enkephalin (figure 32b). A small unidentified peak of EnkLI is present which elutes before Leu- or Met-enkephalin but after the principal oxidized form of Met-enkephalin (figure 32b).

A single peak of GALLI was evident on reverse-phase HPLC which clearly eluted at a retention time different from that of synthetic porcine galanin (figure 33).

On reverse-phase HPLC under 'steep' gradient elution conditions, GRPLI peaks eluted at the retention times of synthetic porcine GRP-(1-27) and its principle oxidized form. Under 'shallow gradient elution conditions, a GRPLI peak eluted close to the retention times of synthetic procine GRP-(1-27) and the second peak close to the elution position of the major oxidized from of GRP-(1-27) and that of GRP-(18-27); the amounts in each peak were similar (figure 35).

The VIPLI in the PV preparations eluted as a single peak of immunoreactivity at the retention time of synthetic porcine VIP on reverse-phase HPLC under 'steep' gradient elution conditions. Employing 'shallow' gradient elution conditions, the VIPLI eluted at the retention time of guinea pig VIP which is clearly later than that of porcine VIP (figure 35).

Cross-reactivity of alpha-NK antiserum with other peptides.

The cross-reactivity of the alpha-NK antiserum with beta-NK, SP, GRP, PYY, Leu-EnK, Met-Enk and VIP was determined. The standard RIA curve for alpha-NK was determined in the presence of alpha-NK or each of the other peptides. The resulting binding displacement curves are shown in figure 36. The alpha-NK antiserum did not cross-react with GRP, PYY, Leu-Enk, VIP or Met-Enk. There was a small cross-reactivity with the tachykinin SP and greater than 50% crossreactivity with the tachykinin beta-NK.

Reverse phase HPLC of tachykinin-like immunoreactivity in the P_2

Lysates of P_2 suspensions were subjected to reverse phase HPLC analysis. The alpha-NKLI and SPLI's of 2 min fractions collected over 90 min, were measured using specific radioimmunoassays. The HPLC chromatograms are shown in figures 37a and 37b. A major peak of alpha-NKLI was evident which co-eluted with standard porcine alpha-NK. A very small but detectable alpha-NK immunoreactive peak was present at the elution time of standard porcine Substance P (figure 37a). A major peak of SPLI which co-eluted with standard porcine SP is shown in figure 37b. A smaller but detectable SP immunoreactive peak was also observed which co-eluted with standard porcine alpha-NK. The ratio of the [alpha-NKLI]/[SPLI] derived from the major immunoreactive peaks was 2.07.

Degradation of exogenous Substance P by P_2 enzymes.

The degradation of 36nM exogenous SP with P_2 synaptosomal

suspensions was measured over a 60 min incubation period following equilibration. A representative degradation curve for SP is shown in fig 39a and the pseudo-first order rate plot of the reaction is illustrated as the inset. Degradation occured rapidly with half of the SP dissappearing in approximately 8 minutes. The rate of degradation measured over the first 20 min of the reaction was about 1600 pmoles/min and the slope of the regression line through the points was -.0770 $\pm$ .0037. The y-intercept of the line was 3.3446 $\pm$ 0.0943 and the correlation coefficient was -0.9965.

The results of a representative experiment showing the rate of degradation of exogenous SP by P_2 enzymes in the presence or absence of various enzyme inhibitors is illustrated in fig 39b. The rate of degradation measured over the first 20 min of the reaction in the presence of 0.5mM bacitracin was 402 pmol/min and the slope through the regression line was -.02686 $\pm$ 0.00019. The y-intercept was 3.5989 $\pm$ .0622 and the correlation coefficient of the line was -.9906. In the presence of a mixture of enzyme inhibitors (0.5mM bacitracin, .25mM leupeptin and 25ug/ml chymostatin) the rate of degradation measured over the first 20 min of the reaction was about 928 pmol/min and this value was significantly different from that obtained either for the control or in the presence of 0.5mM bacitracin alone ($p < .05$). The slope of the regression line was .02374 $\pm$ .00145. The y-intercept of the line was 3.6599 $\pm$ .04880 and the correlation coefficient was -.9930. The rate of degradation of exogenous SP in the presence of bacitracin alone was not

significantly different from that obtained in the presence of the mixture of enzyme inhibitors ($p>.05$). Essentially identical results were obtained in two other experiments performed on separate days; one experiment done using PV (60ug/assay tube) instead of P_2 gave similar results.

Neuronal stores of SPLI in the PV

Following resuspension of the PV in Krebs buffer, substantial depletion of the neuronal content of SPLI occurs over time. The depletion of neuronal content was directly dependent on the equilibration time (figure 40). Less than 40% of the SPLI remains after a 90 min. equilibration period at 37 C. Comparable depletion was also observed with P_2 suspensions. The results represent the mean responses from 4 separate experiments.

Basal Release of SPLI

The basal release of SPLI from P_2 synaptosomes suspended in Hepes buffered medium, decreased with increasing equilibration time as shown in figure 41a. Within 60 min. less than 50% of the initial basal release of SPLI remains. Identical results were obtained with synaptosomal suspensions resuspended in gassed Krebs solution,pH 7.4 (results not shown).

The basal release of SPLI from enteric synaptosomes (P_2) depended on the isolation/resuspension buffer. The highest amount of basal release of SPLI was obtained from synaptosomes isolated in sucrose

(0.32M) and resuspended in either gassed Krebs or Hepes buffered medium. By comparison, significantly lower amounts of basal release were measured in suspensions which were isolated and resuspended in either Krebs or Hepes buffered medium ($p < .05$) [Basal release in these experiments was measured in the presence of 5mM bacitracin].

Effect of bacitracin on basal release of SPLI.

Inclusion of the enzyme inhibitor bacitracin in the incubation mixture with the synaptosomes (P_2), dose-dependently enhanced the basal release of SPLI detectable (see figure 42a). When synaptosomes were resuspended in Krebs buffer (gassed with 95%O_2/5%CO_2; pH 7.4), a linear increase in basal release was evident with incrementing concentrations of bacitracin. The correlation coefficient of the linear regression line is 0.9934, the x-intercept is 0.529, the y-intercept is -116 $\pm$ 50.9 and the slope is 219.5. No plateau phase was evident with concentrations of bacitracin as high as 5mM. A dose-dependent increase in basal release reaching a plateau around 2.5mM bacitracin was obtained with samples resuspended in Hepes buffered medium (pH 7.2).

In the presence of 5mM bacitracin the amount of basally released SPLI which can be detected is increased by 129% $\pm$ 5% from control levels measured in the absence of bacitracin (figure 42b).

Concentration of cations present in the extracellular environment of the synaptosomes.

The extracellular levels of $[K^+]$, $[Ca^{++}]$ and $[Mg^{++}]$ were measured in the S_3 and S_4 supernatants (see methods for details of methodology). The results are presented in figure 43a and table 12. Figure 44a shows the standard curve used for determining the amount of $[Ca^{++}]$ that was present in the supernatants. Significantly lower levels ($p < .05$) of $[K^+]$ and $[Mg^{++}]$ were detected in the S_4 compared with those found in the S_3. The amounts of all cations detected in both supernatant fractions were significantly lower than those used routinely in the resuspension buffer. For example, the amounts of $[K^+]$ and $[Ca^{++}]$ were approximately 10 fold and 85 fold lower, respectively, than those used in the resuspension buffer.

The concentration of $[Ca^{++}]$ measured in the S_3 fraction was 29.0 ± 3.0 uM.

Sample blanks for several neuropeptides contained in myenteric varicosities (P_2).

P_2 sample blanks for alpha-NKLI, MELI and SPLI were determined for a range of dilutions and the results are shown in figures 38a to 38c; relatively high sample blank B/F ratios (i.e. B/F > .3) were obtained for both alpha-NKLI and MELI, respectively, with more concentrated P_2 samples (i.e. P_2 dilution < 20 fold). Therefore, in all subsequent experiments, the alpha-NKLI and the MELI were measured in P_2 samples diluted greater than 25 fold. The sample

blank B/F ratios for SPLI are as low as the standard curve blanks irrespective of the dilution of the sample (figure 38c).

Effect of centrifugation speed on basal release of SPLI and alpha-NKLI from enteric synaptosomes (P_2).

The basal release of both SPLI and alpha-NKLI from P_2 synaptosomes is dependent on the centrifugation speed used to separate free peptide (supernatant) from that which is still contained in the varicosities (pellet) (see figure 44). With centrifugation of the S_3 supernatant at 105,900 X g, the resulting S_4 supernatants contained approximately 75% of both the initial S_3 SP and alpha-NK - like immunoreactivities. The remaining 25% of the tachykinin immunoreactivities were present in the P_u pellets obtained with the high speed centrifugation.

P_2 sample blanks for tachykinins present in supernatants obtained with different centrifugation speeds.

The sample blank B/F ratios for alpha-NKLI were the same in the S_3 and S_4 supernatants over the sample dilution range employed (see figure 45b. The P_u pellet did not contribute to any of the binding observed with the alpha-NK tracer. Binding of the SP tracer to the S_3, S_4 or P_u fractions was essentially zero.

Release of tachykinins with miscellaneous substances

The adenylate cyclase stimulator forskolin (50uM) (n=4) and the the calcium ionophore A23187 (50uM) (n=4) failed to produce

detectable release of alpha-NK and SP-like-immunoreactivities from the synaptosomes. The sodium ionophore monensin dose-dependently evoked release of both SPLI and alpha-NKLI from the same preparations. A representative experiment is shown in figure 46.

Basal release of peptide-immunoreactivities from P_2 synaptosomes.

The basal release of several peptide-immunoreactivities present in the P_2 is presented in table 14. The basal release of peptide-immunoreactivities, in order of highest to lowest, is MELI > SPLI > alpha-NKLI > LELI > GALLI; the neuronal content of the peptides is in the order of SPLI >/ alpha-NKLI = MELI >> LELI > GALLI. From table 14, it is clear that enkephalins (LELI and MELI) have both the highest basal and evoked release in terms of their respective neuronal contents in the P_2, in comparison to the tachykinins (alpha-NKLI and SPLI).

Effect of K^+-evoked depolarization on release of neuropeptide immunoreactivities from enteric varicosities (P_2).

The effect of K^+-depolarization on the release of SP-like immunoreactivity (LI), alpha-NKLI, Met-ENK-LI, Leu-ENK-LI and GAL-LI was measured in the same P_2 synaptosomal preparations.

i) Tachykinin-like immunoreactivity

Release of alpha-NKLI and SPLI from enteric synaptosomes was not detectable with K^+-depolarization (10 to 100mM $[K^+]$)(results from 6

separate experiments confirmed this finding) in the absence of antagonists. Subsequent experiments were carried-out in the presence of several different antagonists of recognized endogenous inhibitors. In the presence of 20uM naloxone, 5uM atropine, 10uM DPSPX or a mixture of all three antagonists in the P_2 suspension, K^+- depolarization (10-100mM) failed to produce detectable release of SPLI. The results are plotted in figures 47 and presented in table 13. In the presence of naloxone, atropine or the mixture, K^+-depolarization failed to produce detectable release of alpha-NKLI (see figure 48). In the presence of DPSPX (10uM) K+-evoked release of alpha-NKLI was clearly detectable (see figure 49). The net evoked release of alpha-NKLI was 1810 $\pm$ 420 fmol/mg protein (The intra-assay variability between replicate values was < 5%).

ii) Enkephalin-like immunoreactivity

In the presence of either the opioid receptor antagonist naloxone (20uM) or the muscarinic receptor antagonist atropine (5uM), K^+-depolarization clearly evoked release of Met-enkephalin-like immunoreactivity. With suspensions containing atropine, increasing K^+ concentrations resulted in a biphasic release response in two experiments; this consisted of an initial decrease in the release of MELI followed by a steady dose-dependent increase in release. The results from three separate experiments at 3 different sample dilutions are presented in figures 50a and 50b. Release with K^+-depolarization was not unequivocally observed in the presence of either the adenosine receptor antagonist DPSPX (10uM) (figure 50c)

or the mixture of DPSPX (10uM), naloxone (20uM) and atropine (10uM) (figure 50d).

Consistent release of Leu-ENK-LI was clearly observed in the presence of the antagonists atropine, DPSPX and naloxone (figure 51a). In the presence of atropine (figure 51b) or naloxone (figure 51c) alone, release was also observed although less prominent. Release in the presence of DPSPX alone was clearly evident in one experiment (figure 51d).

In these same experiments, K^+-depolarization failed to produce detectable release of GAL-LI in the presence or absence of any of the antagonists. The results are plotted in figure 47.

The basal release of neuropeptide-like immunoreactivities in order of highest to lowest, observed in the same experiments, was: Met-ENK > SP = alpha-NK > LE > GAL -like immunoreactivity (figs 47, 48, 49, 50).

Calcium dependence of the basal release of neuropeptides from enteric nerve endings.

Incrementing concentrations of extracellular $[Ca^{++}]_o$ had no effect on the basal release of SP, alpha-NK or Met-ENK-like immunoreactivities from enteric nerve endings. Results from a representative experiment are shown in figures 52a, 52b, 52c, respectively. The calcium channel blocker D600 (100uM) clearly

inhibited a portion of the basal release of these neuropeptide-entities. Essentially identical results were obtained in one additional experiment.

Effects of Gastrin Releasing Polypeptide (GRP) on atropinized LMMP.

GRP dose-dependently contracted the atropinized LMMP preparation (figure 53a). Bombesin (1uM) was less potent than 1uM GRP at contracting the LMMP strip ($p<.05$).

The GRP-mediated contraction was almost abolished by the Na^+ channel blocker TTX (5uM) ($p<.05$) and partially inhibited by 40uM of the SP antagonist [d-Pro^4,$Trp^{7,9,10}$] SP (4-11) ($p<.05$). The results are presented in figure 53b.

Release of neuropeptides from isolated myenteric varicosities (P_2) with Gastrin Releasing Polypeptide.

Gastrin releasing polypeptide dose-dependently evoked release of both alpha-NK and SP-like-immunoreactivities from the same synaptosomal preparations. The results are presented in figure 54a and 54b respectively. Clearly, on a molar basis, GRP releases more alpha-NKLI than SPLI from enteric varicosities. The ratio of alpha-NK : SP released by GRP is approximately 2.5 : 1. The neuronal contents of these tachykinins are in the ratio of approximately 1.0 : 1.0. Figures 54a and 54b also illustrate the dose-dependent release of alpha-NK and SP-like immunoreactivities with bombesin.

Bombesin is less potent at releasing the tachykinins than GRP. Bombesin releases more alpha-NKLI than SPLI from enteric varicosities. The ratio of alpha-NK to SP released by bombesin is approximately 2:1. Essentially identical results were obtained in two other experiments.

In a single experiment (figures 55a and 55b), the release of both alpha-NK and SP-like immunoreactivities were shown to be greater at a 60 minutes than at 30 min equilibration.

Both GRP and bombesin dose-dependently evoked release of GALLI from enteric synaptosomes. A representative experiment is shown in figure 56. It appears that Bombesin is more potent than GRP at releasing GALLI from the synaptosomes (experiments repeated two more times with essentially the same results).

The highest concentrations of GRP (50uM) and bombesin (100uM) did not produce maximum release of the neuropeptides.

In single preliminarly experiments, the presence of 20uM naloxone in the incubation mixture did not alter the potency of GRP in releasing either of the tachykinins. An experiment with the putative GRP receptor antagonist [Leu^{13}-psi-CH_2NH-Leu^{14}]-bombesin (50uM) failed to shift the dose response relationship of GRP (1nM to 50uM range) at releasing the tachykinins.

In 3 preliminary experiments, the selective A_1 adenosine receptor agonist CPA had no effect on basal or GRP-mediated release of alpha-neurokinin or substance P - like immunoreactivities.

TABLE 1.

EFFICACY OF NUCLEOSIDES AND AFFINITY OF ADENOSINE RECEPTOR ANTAGONISTS AT THE STIMULATED ILEUM (0.2Hz,1msec).

COMPOUND	EC_{50} (M)	pA_2 of THEOPHYLLINE	pA_2 of DPSPX
R-PIA (9)[a]	2.6×10^{-8}M[b]	4.90	---
CHA (10)	1.2×10^{-8}M	5.22	6.74
NECA (12)	1.7×10^{-8}M	5.04	6.86[c]
CPA (8)	5.0×10^{-8}M	4.89	6.72
MCPA (8)	4.48×10^{-8}M	---	6.68

[a] Number in parentheses, number of separate tissues used.

[b] S.E.M. of EC_{50} values $\pm$ 6.0%

[c] Apparent pA_2.

TABLE 2.

ANTAGONISM BY [d-Pro^{4},d-Trp^{7,9,10}] SP (4-11) OF AGONIST-MEDIATED CONTRACTIONS OF THE ATROPINIZED GUINEA PIG LMMP.

		RESPONSE	
Agonist	% of contraction to 5nM CCK8	+ 5uM tetrototoxin	+ 40uM SPA
CCK8 5nM	100	1.0±0.7**	0.0**
SP 5nM	100	100	0.0**
ES (20Hz;.75ms)	110±8.0	0**	35.0±6.0*
KCl 10mM	91.0±9.4	91.0±9.4	80.6 ± 7.5
HIST. 50 uM	77.6±9.0	42.6±8.2*	30.0±10.1*

± S.E.M. from 4-8 separate LMMP preparations.

* significant difference from control at .05 level.

** significant difference from control at .01 level.

TABLE 3.

INHIBITION OF TACHYKININ - MEDIATED CONTRACTIONS OF THE LMMP BY ADENOSINE ANALOGS.

ADENOSINE ANALOG	5nM CCK8 RESPONSE ** (EC_{50})	FIELD STIMULATION (EC_{50})
NECA (6)	$3.4 \pm .4 \times 10^{-8}$M	(7) $2.C \pm .48 \times 10^{-8}$M (63.5% $\pm$ 6.1)*
CPA (6)	$2.4 \pm .4 \times 10^{-9}$M	(5) $1.8 \pm .74 \times 10^{-8}$M (67.6% $\pm$ 3.8)*
2PAA (4)	$2.2 \pm .46 \times 10^{-6}$M	

$\pm$ S.E.M.'s calculated from data obtained from 4 to 7 preparations as indicated in parantheses; r values > 0.90 for individual regression lines used to obtain EC_{50}'s.

* note that the EC_{50} values for field stimulated responses were calculated from the maximum inhibition produced by each analog as reported in parentheses.

** nucleosides produced 100% inhibition of the responses.

Table 4.

COMPARISON OF YIELDS AND OCCLUDED LDH UNITAGE FOR VARIOUS FRACTIONS OBTAINED DURING THE ISOLATION OF PV FROM GUINEA PIG ILEAL LMMP STRIPS.

Fraction	Yield	Occluded LDH
	ug of protein/animal*	units/mg of protein
P_2	1241 ± 176 (n=3)	343 ± 83 (n=3)
Mitochondrial	1490 ± 130 (n=4)	68 ± 25 (n=4)
PV	178 ± 26 (n=11)	1312 ± 85 (n=11)

* Mean ± S.E.M.

TABLE 5.

COMPARISON OF AFFINITY OF NUCLEOSIDES AT PV WITH THEIR BIOLOGICAL ACTIVITY AT THE STIMULATED ILEUM

LIGAND	DISPLACEMENT OF LABELED NUCLEOSIDE (IC_{50})		INHIBITION OF STIMULATED ILEUM PREPARATION	
	$[^3H]$R-PIA	$[^3H]$NECA	EC_{50}*	pD_2**
Competitor				
R-PIA	$1.9X10^{-8}M$	---	$2.5X10^{-8}M$	7.60
NECA	---	$2.2X10^{-7}M$	$1.6X10^{-8}M$	7.79
2-Chloroadenosine	$1.1X10^{-8}M$	$3.3X10^{-7}M$	$1.8x10^{-8}M$	7.74
Adenosine-N^1-oxide	$5.6X10^{-7}M$	$1.4X10^{-6}M$	$8.3X10^{-7}M$	6.08
Purine riboside	$2.9X10^{-5}M$	$9.5X10^{-4}M$	$2.3X10^{-5}M$	4.64
Inosine***	$1.3X10^{-4}M$	$4.3X10^{-4}M$	$2.2X10^{-4}M$	3.65

* Determined on a minimum of three preparations. S.E.M.= $\pm$ <10%.

** calculated as $-Log(EC_{50})$

*** e = 0.7.

TABLE 6.

COMPARISON OF IC_{50} VALUES FOR DISPLACEMENT OF LABELED NUCLEOSIDES BY VARIOUS COMPETITORS.

COMPETITOR	DISPLACEMENT OF RADIOLIGAND	
	[^{3}H]CHA	[^{3}H]NECA
	IC_{50}	IC_{50}
R-PIA	----	($1.0X10^{-4}M$,42%)[a]
CHA	$2.2X10^{-7}M$	($1.0X10^{-4}M$,38%)
NECA	$3.5X10^{-7}M$	$2.2X10^{-7}M$
DPSPX	$1.2X10^{-5}M$	($5.0X10^{-4}M$,52%)
THEOPHYLLINE[b]	----	$1.3X10^{-4}M$

[a] Where 100% displacement of the specific binding of the radioligand was not achieved, the values in parentheses are the maximum concentrations used and the percentage of displacement achieved.

[b] Determined in three to five separate experiments. S.E.M. $\pm$ < 10%.

TABLE 7.

SATURATION BINDING CHARACTERISTICS OF ^{3}H-[NECA] ON SUBCELLULAR PV AND MITOCHONDRIAL FRACTIONS ISOLATED FROM STRIPS OF GUINEA PIG LMMP.

Parameter	MITOCHONDRIAL FR.	PURIFIED VARICOSITY FR.
K_d	$1.0 \pm .37 \times 10^{-7}$M *	$3.4 \pm 1.4 \times 10^{-8}$M
B_{max}	2.4 ± 1.322 pmoles/mg	1.5 ± 0.47 pmoles/mg
Hill Coeff.	.97 ± .025	.99 ± .066

* ± represents the means from 3 separate experiments.

Data analyzed using EBDA & SCATFIT programs.

TABLE 8.

SATURATION BINDING CHARACTERISTICS OF ^{3}H-[CHA] ON SUBCELLULAR PV AND MITOCHONDRIAL FRACTIONS ISOLATED FROM STRIPS OF GUINEA PIG LMMP.

Parameter	MITOCHONDRIAL FR.	PURIFIED VARICOSITY FR.
K_d	$4.4 \pm .4 \times 10^{-8}$M *	$2.03 \pm 1 \times 10^{-8}$M
Bmax	2.93 ± .215 Pmoles/mg	0.335 ± .190 Pmoles/mg**
Hill Coeff.	.923 ± .015	.987 ± .076

* ± represents the means from 3 separate experiments.

Data analyzed using EBDA & SCATFIT programs.

** significantly different from Bmax on MIT FR. at .05 level.

TABLE 9.

SPECIFIC BINDING OF [^{3}H]NECA TO VARIOUS FORMS OF THE PV.

FRACTION	TOTAL BINDING (pmoles/mg)	NON SPEC. BINDING[a] (pmoles/mg)	SPEC. BINDING (as % total bind.)
Intact PV	0.765 ± .095	0.351 ± .005	54.1
Membranes	*1.075 ± .032	0.551 ± .08	45.8
Boiled PV	**0.358 ± .063	0.496 ± .165	0.0[aa]

[a] Nonspecific binding determined in the presence of 50uM NECA

[aa] significantly lower ($p<.001$) than specific binding at intact PV (in the presence of 100uM NECA the displaceable binding is 63.8%)

* total binding is significantly higher ($p<.05$) than that at intact PV

**total binding is significantly lower ($p<.05$) than that at intact PV and equal to the non-specific binding at intact PV ($p>.05$).

TABLE 10.

NEUROPEPTIDE CONTENT IN PV PREPARATIONS

Peptide	Content (pmol/mg protein)*
SPLI	74.2 ± 11.2
VIPLI	3.4 ± 0.8
GalLI	1.2 ± 0.3
GRPLI	3.3 ± 0.8
MELI	35.3 ± 8.2
LELI	4.9 ± 0.6

* Mean ± S.E.M. of immunoreactive quantitities measured in 7 PV preparations.

TABLE 11.

NEURONAL PEPTIDE CONTENT IN VARIOUS FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES CONTAINING MINCED GUINEA PIG ILEAL LONGITUTINAL MUSCLE - MYENTERIC PLEXUS.

			[NEUROPEPTIDE] (fmol/G.P.)*			
FRACTION	YIELD (mg/G.P.)	occl.LDH U per G.P.	[GRPLI]	[MELI]	[LELI]	[VIPLI]
LMMP	3,590 ±200	---	---	---	---	---
P_2	5.44 ±.37	1264 ±135	2052 ±163	39,143 ±2,782	10,070 ±742	3.676 ±239
F_a	.202 ±.03	10.34 ±5.7	334.5 ±29.2	1711 ±390	1783 ±239	415 ±78
F_b	.640 ±.10	185.0 ±25.3	618.2 ±38.6	1892 ±386	2630 ±233	723 ±41
F_c	.915 ±.11	113.4 ±25	279.8 ±16.4	479.5 ±23.3	817 ±130	435 ±80
$[P_2]/[F_b]$	---	---	3.32 ±.23	20.7 ±2.9	3.83 ±.31	5.08 ±.31

* Mean ± S.E.M. of 4 separate experiments.

Table 12.

Measurement of the extracellular levels of $[Ca^{++}]_o$, $[Mg^{++}]_o$ and $[K^+]_o$ in the S_3 and S_4 supernatant fractions of P_2 suspensions.

[Cation] (uM)	*S_3 supernatant	*S_4 supernatant
K^+	475	82.1
Ca^{++}	29.6	41.7
Mg^{++}	225	5.83

* the mean values from 4 separate experiments are presented; the standard error of the means is < 5%.

TABLE 13.

EFFECT OF THE DEPOLARIZING STIMULUS K+ ON THE RELEASE OF SPLI FROM CRUDE MYENTERIC VARICOSITIES (P_2).

	[SPLI] (fmols/ml)* in the presence of antagonists			
K+ (mM)	NAL. (20uM)	ATR.(10uM)	DPSPX (10uM)	ALL 3 ANTAG.
0	1961±75**	1797±161	1801±122	1860±140
1	1958±111	2057±18	2019±96	1886±111
5	2036±51	2046±55	1990±87	1881±111
10	2096±58	1909±79	1913±95	1632±150
20	2042±33	2007±2	1895±162	1798±134
30	2111±214	2004±96	2004±48	1848±75
50	2027±34	2034±15	1855±163	1865±161
75	1987±57	1907±204	1921±104	1967±42
100	1991±180	2000±186	1928±196	1926±121

* each ml of incubation contained 0.7±.05mg P_2 protein.

** ± represents the S.E.M. from 3 separate experiments.

Table 14. Release of peptide-immunoreactivities from P_2 synaptosomes.

Peptide	Basal Rel. (fmol/mg protein)	K^+-depol. (Net rel.)	content	Basal Rel. (% Cont.)	K^+-depol. (% Cont.)
a-NKLI	3577±154	1810±420	27326±930	13.0	7
SPLI	4516±173	0	31101±777	14.5	0
MELI	8333±1472	9778±2762	25000±3500	33.3	39
LELI	850±102	1000±386	3772±278	23	27
GALLI	undetected	undetected	----	----	--

± S.E.M. for pooled data derived from 3 separate experiments. For a-NKLI, K+-evoked release measured in presence of DPSPX (10uM); for SPLI, measured in presence of naloxone (20uM); for MELI, measured in presence of naloxone (20uM); for LELI, measured in presence of naloxone (20uM), atropine (5uM) and DPSPX (10uM).

Figure 1

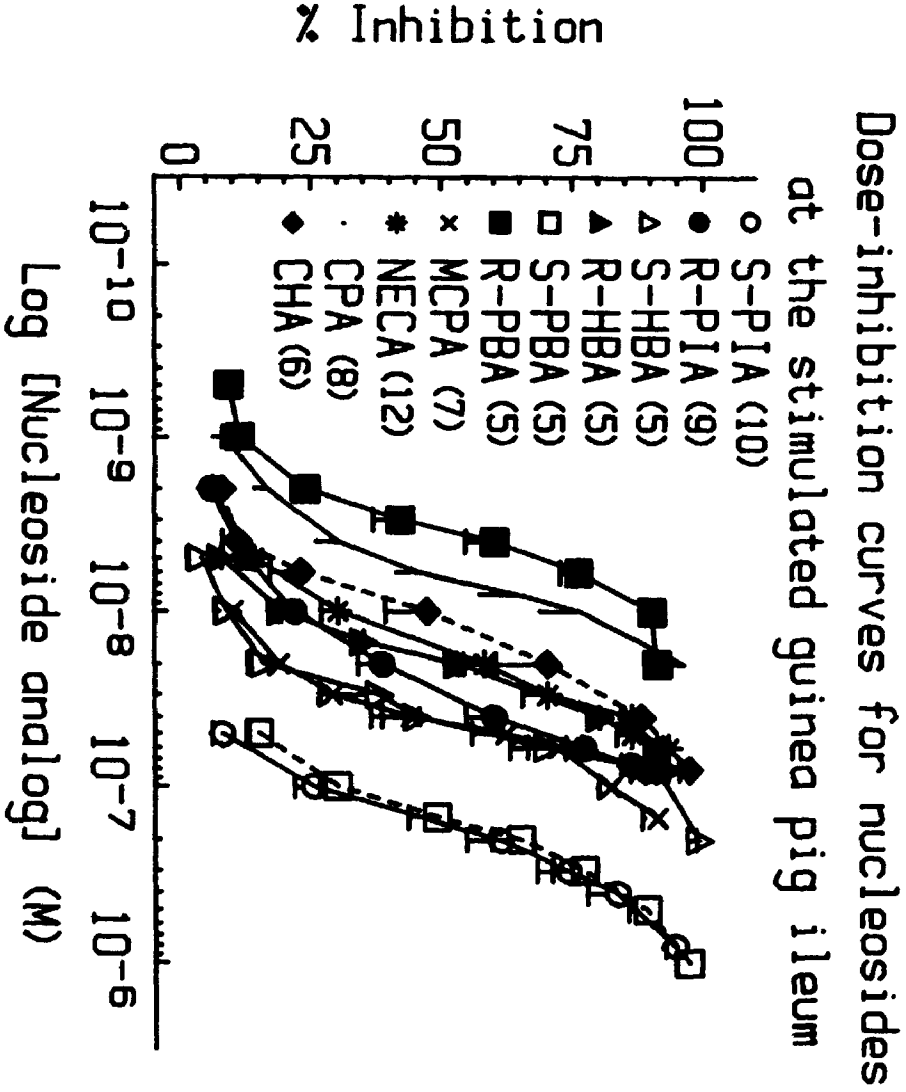


Fig. 1. Dose-inhibition curves obtained on the stimulated ileum preparation to the stable nucleoside analogs S-PIA, R-PIA, S-HBA, R-HBA, S-PBA, R-PBA, MCPA, NECA, CPA and CHA. Each point represents the mean response from the number of preparations listed in parentheses. The error bars represent the S.E.M.'s.

Fig. 2. Dose-inhibition curves obtained on the stimulated ileum preparation to the R- and S-diastereoisomers of the N^6-substituted nucleoside analogs a) HBA, b) PIA and c) PBA. Each point represents the mean response from 5 to 8 preparations. The standard errors are $\pm < 6.0\%$

Figure 2

BIOLOGICAL ACTIVITIES OF SEVERAL ENANTIOMERIC PAIRS OF N⁶-SUBSTITUTED ANALOGS OF ADENOSINE

Fig. 3. Schild plots derived from EC_{50} values for a) NECA, b) R-PIA, c) CHA and d) CPA obtained in the absence and presence of incrementing concentrations of theophylline. The isoboles are shown as the least squares regression lines (r^2 >.96) and the x-intercepts indicate the affinity constant(s) (pA_2) of the antagonist. Values are means of four to six experiments.

Figure 3

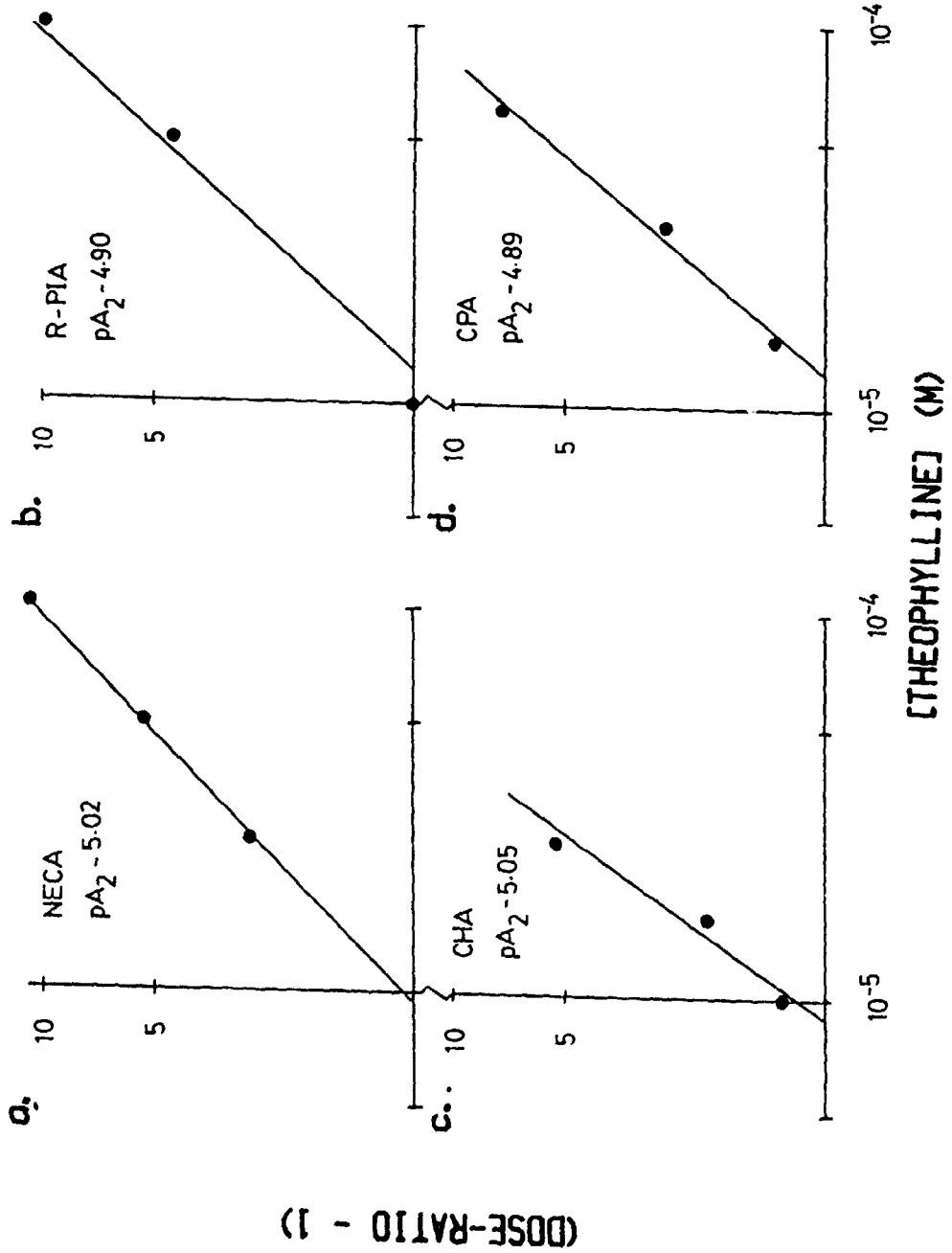

a.
NECA
pA_2 - 5.02
b.
R-PIA
pA_2 - 4.90
c.
CHA
pA_2 - 5.05
d.
CPA
pA_2 - 4.89
10
5
10^{-4}
10^{-5}
(DOSE-RATIO - 1)
[THEOPHYLLINE] (M)

Fig.4. Schild plots derived from EC_{50} values for CHA, MCPA and NECA obtained in the absence and presence of incrementing concentrations of DPSPX. The isoboles for CHA and MCPA are shown as the least-squares regression lines (r^2 for CHA = 0.995, for MCPA = 0.999) and the pA_2 values are 6.74 for CHA and 6.68 for MCPA. Values are means of four to six experiments. *, significant differences of the confidence intervals about the data points for NECA compared with CHA or MCPA. The isobole for NECA was constructed by joining the points. Bars are S.E.M. Schild plots derived from EC_{50} values for CHA and NECA obtained in the presence and absence of incrementing concentrations of theophylline. The isoboles are shown as the least squares regression lines (r_2 for CHA = 0.993, for NECA = 0.999) and the pA_2 values values are 5.22 for CHA and 5.04 for NECA. Values are means of four to six experiments.

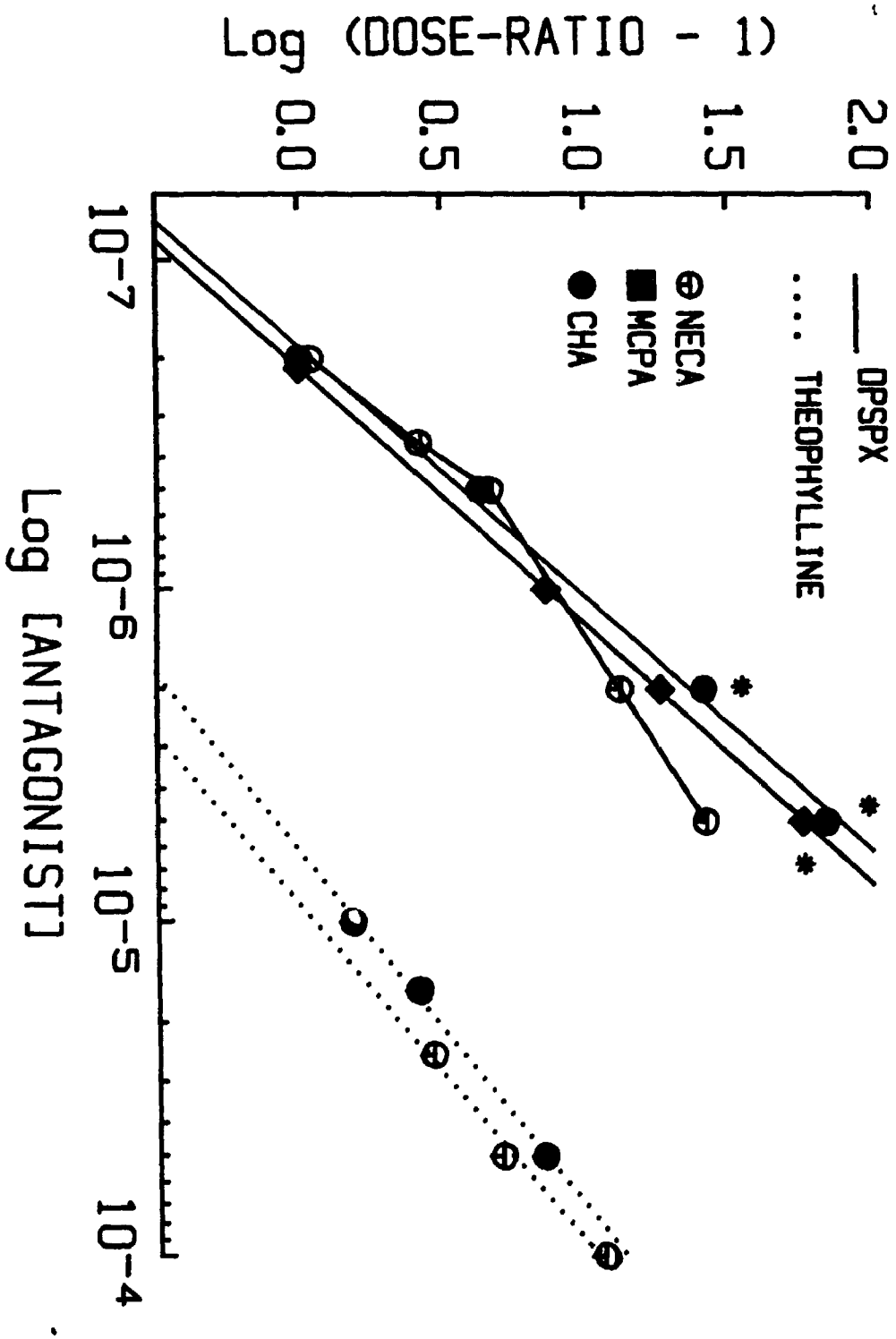

Log (DOSE-RATIO - 1)
0.0
0.5
1.0
1.5
2.0
Log [ANTAGONIST]
10-7
10-6
10-5
10-4
DPSPX
THEOPHYLLINE
NECA
MCPA
CHA

Figure 4

Fig. 5. a) Tracings showing typical contractile responses to 5nM CCK8 and 5nM SP in the presence or absence of the substance P antagonist [D-Pro^{4},Trp^{7,9,10}] SP (4-11) (SP-A) obtained at the atropinized (2uM) LMMP strip preparation. b) Tracings showing the contractile responses of the atropinized LMMP preparation to electrical stimulation at 20Hz and 0.75msec pulse duration (S_1,S_2,$S3$) in the presence and absence of incrementing concentrations of the SP-A. These contractile responses were reproducible in at least 3 other experiments.

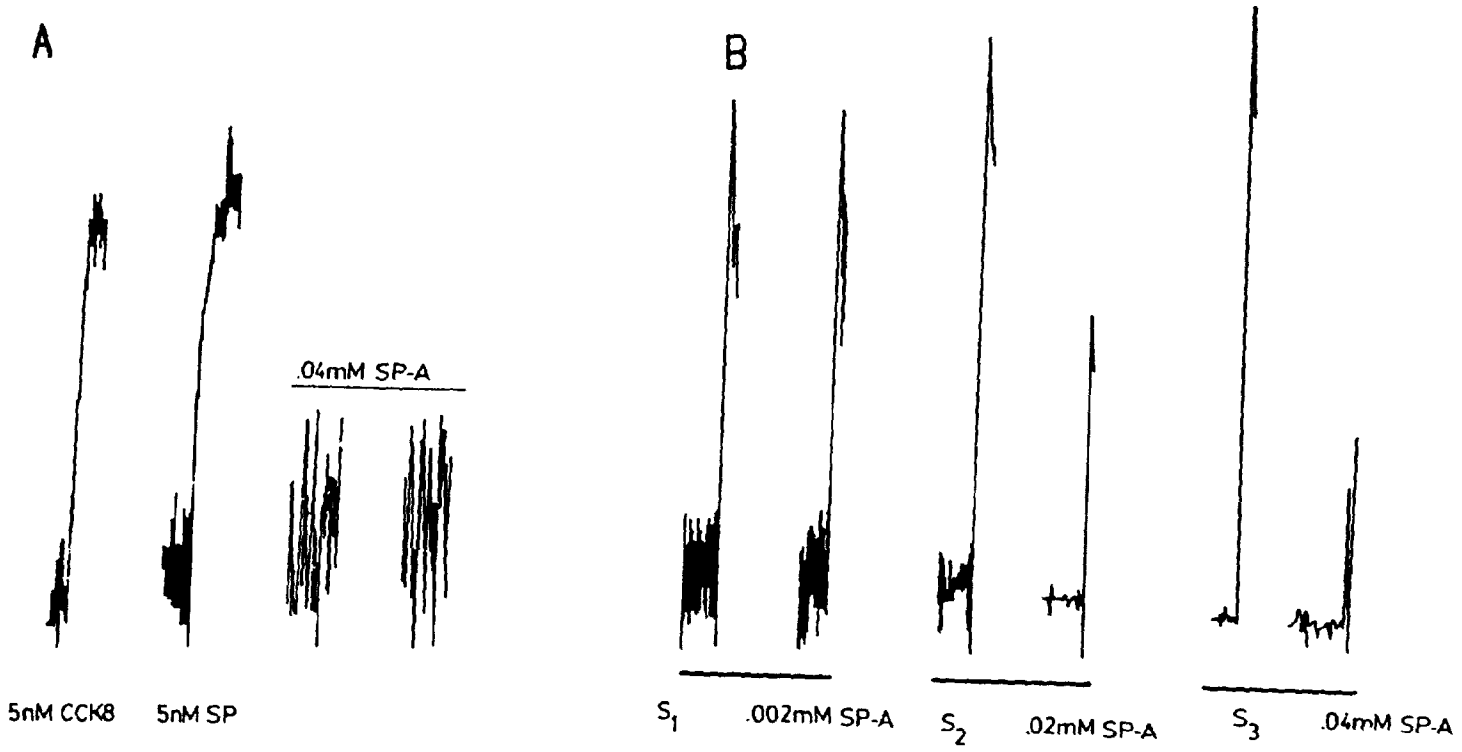


Figure 5

Fig. 6. Tracings from a typical desensitization experiment to substance P. Following electrical stimulation (20Hz,.75msec pulse duration) of the atropinized (2uM) LMMP strip, SP(0.5uM) was added to the Krebs medium bathing the tissue and washed 15 min. later. This cycle was repeated 3 times over 45 min. at which time test doses of 50uM histamine or ES added to the bath failed to produce any effect. The recovery of the tissue following several successive washes at 5 min intervals is shown with the responses to ES slowly returning to control levels. The atropinized preparation did not respond to 1uM Ach. This experiment was repeated once with similar findings.

Figure 6

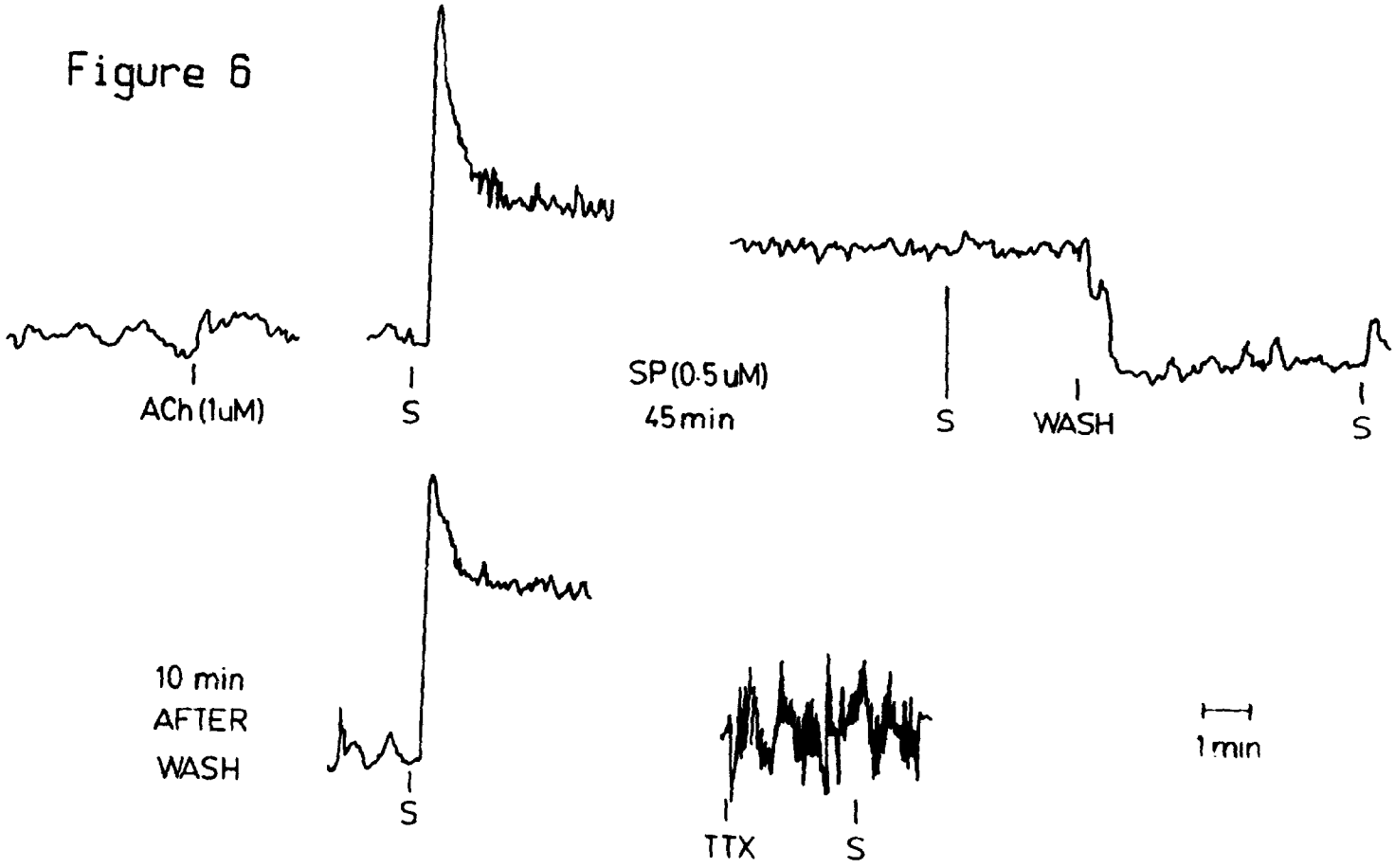


Fig. 7. Dose-inhibition curves to nucleoside analogs obtained on the atropinized (2uM) guinea pig ileum LMMP. a) Dose-response curves obtained on CCK8-mediated (5nM) contractions of the LMMP to CPA, NECA and 2PAA. b) Dose-response curves obtained on the electrically stimulated (20Hz,.75msec.) preparation to CPA and NECA. Each point represents the mean response from 4 to 6 preparations. Bars are S.E.M.

Figure 7

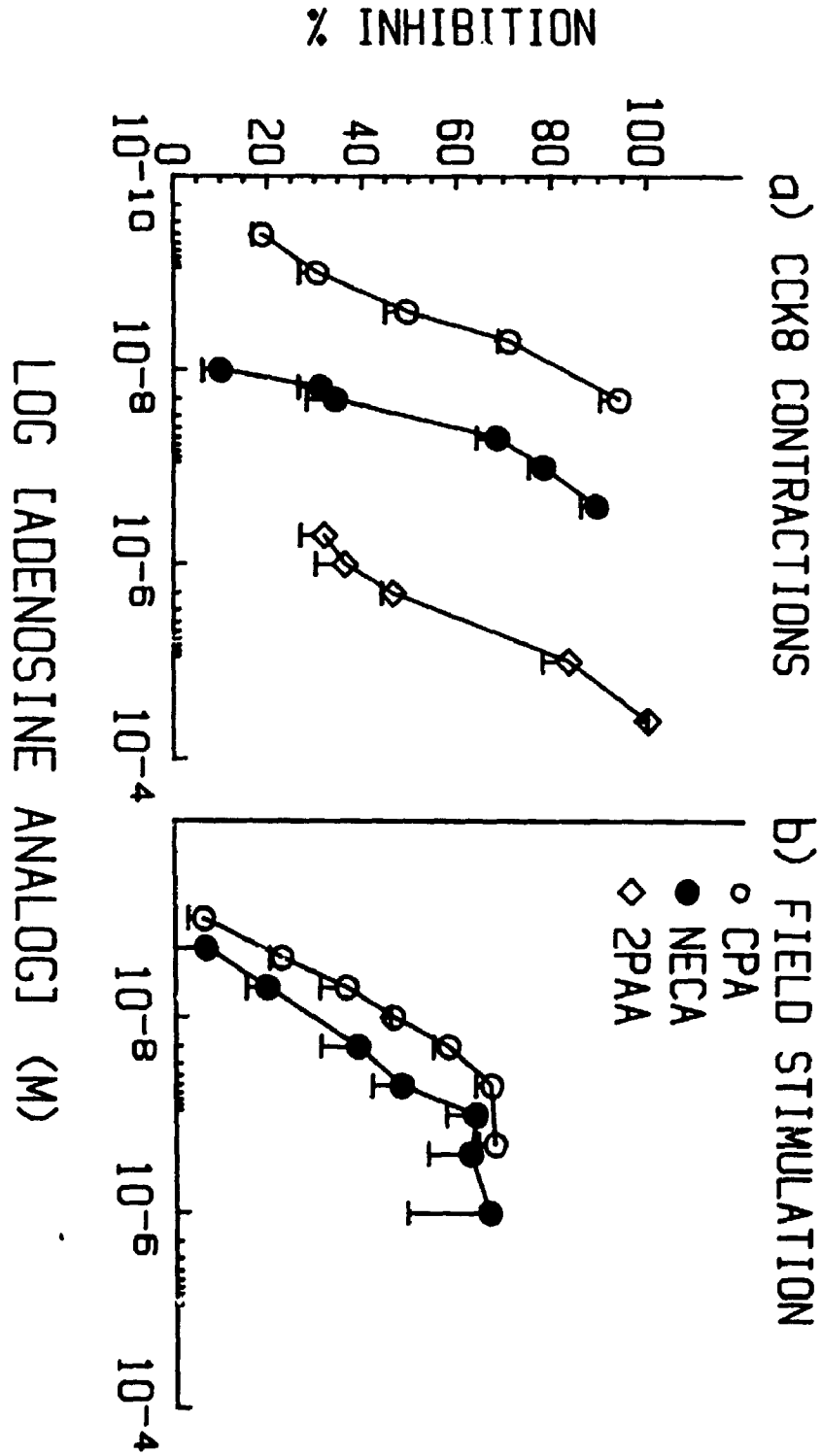

% INHIBITION
0
20
40
60
80
100
a) CCK8 CONTRACTIONS
b) FIELD STIMULATION
○ CPA
● NECA
◇ 2PAA
10⁻¹⁰
10⁻⁸
10⁻⁶
10⁻⁴
LOG [ADENOSINE ANALOG] (M)

Fig. 8. Schild plots obtained at the atropinized LMMP strip, derived from EC_{50} values for selective adenosine analogs obtained in the absence and presence of incrementing concentrations of A_1 selective adenosine receptor antagonists a) The isobole for NECA against CPDPX is shown as the least-squares regression line (r^2 = 0.990) and the pA_2 value CPDPX is 8.0; b) Represents the isobole for NECA against DPSPX (r^2 = .970) with a pA_2 value for DPSPX of 6.52. c) Represents the isobole for NECA against DPSPX (r^2 = .999) with a pA_2 value for DPSPX of 7.0. Values are means of at least four experiments.

Figure 8

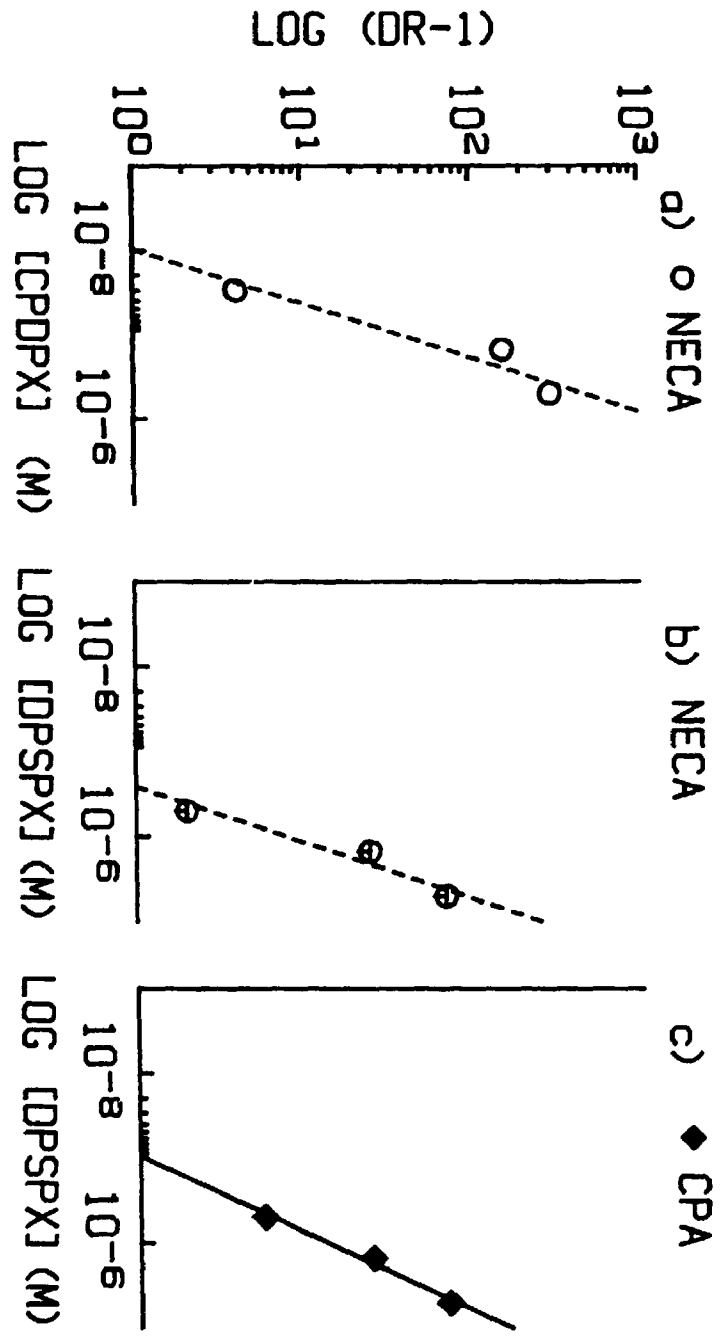

LOG (DR-1)
10^0
10^1
10^2
10^3
a) o NECA
LOG [CPDPX] (M)
10-8
10-6
b) NECA
LOG [DPSPX] (M)
c) ◆ CPA

Fig. 9a. Relationship between the occluded LDH activity obtained for PV preparations and the fraction of total binding that was displaceable. Assay volume was 300 ul and incubation was for 12 mins. at 22 C. [^{3}H]-NECA and [^{3}H]-R-PIA were the radioligands used. Each point on the diagram represents the mean response from at least 4 separate experiments which yielded values within < 5% of each other. Collectively the data represents the findings from more than 50 experiments.

Figure 9a

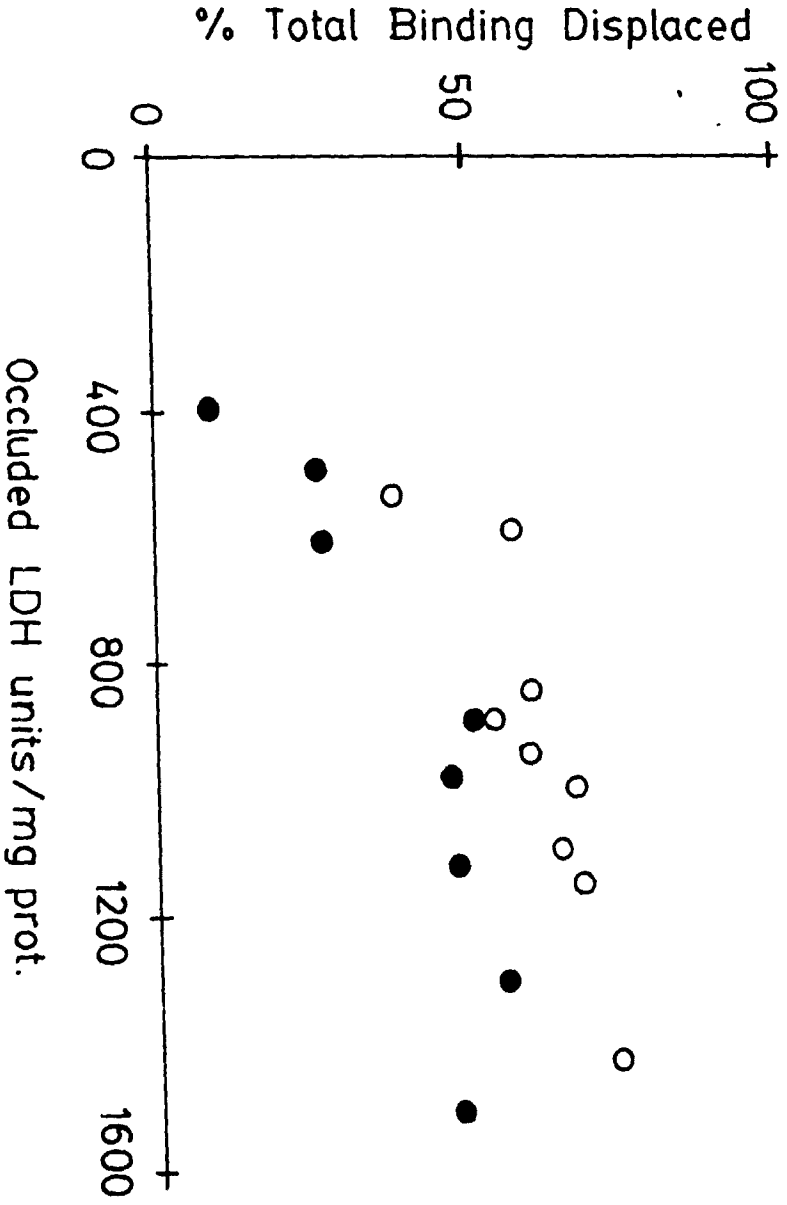

% Total Binding Displaced
0
50
100
0
400
800
1200
1600
Occluded LDH units/mg prot.

Fig. 9b. Electron micrographs showing various types of terminal varicosities (V) present in the purified varicosity fraction. Vesicles with different morphologies may be seen within the varicosities (arrows) including large granular vesicles (A), small agranular vesicles (C) and large dense cored vesicles with an electron-lucent halo (D). Mitochondria (M) are seen within several varicosities while free mitochondria (A) are rare. Membranous profiles with electron-lucent centres (G) are also present. Bars on each micrograph = 0.5 um.

Figure 9b

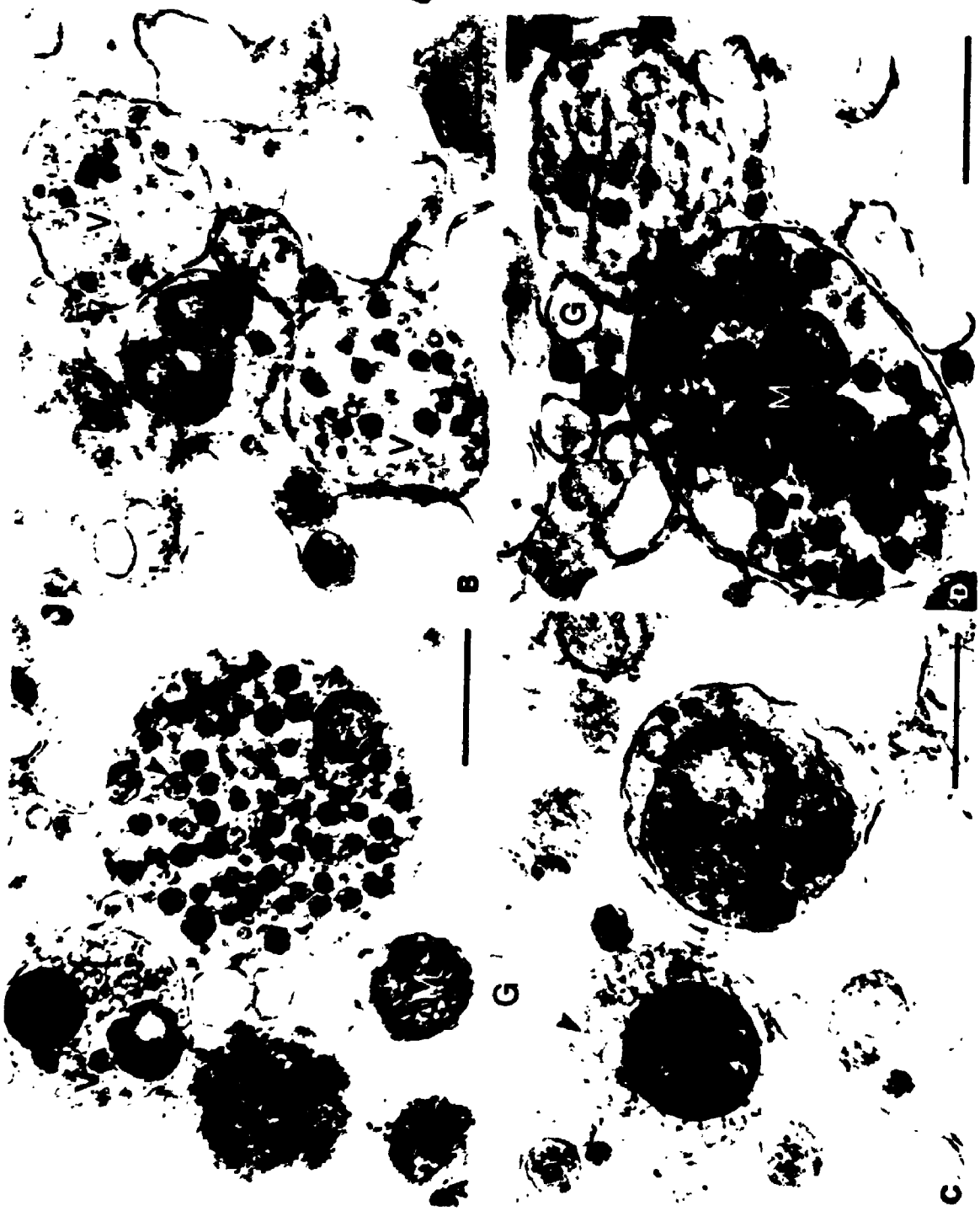


Fig. 10. Displacement curves showing specific binding of labeled nucleoside ligands to PV in the presence of various concentrations (molar) of unlabeled competitors.

a) Displacement curves showing specific binding of [^{3}H]-NECA (2 X 10^{-8} M) to PV. The maximum displacement of [3H]NECA by its unlabeled counterpart was taken as 100% specific binding. Displacement by other unlabeled competitors was related to this value. Values are means of five (NECA), three (2-Cl-Aden), four (Pur Rib), four (Ad-N1-Ox) and four (Ino), experiments respectively. S.E.M. values were $\pm$ < 15%.

b) Displacement curves showing specific binding of [^{3}H]-R-PIA (5 X 10^{-9} M) to PV. The maximum displacement of [^{3}H]-R-PIA by its unlabeled counterpart was taken as 100% specific binding. Displacement by other unlabeled competitors was related to this value. Values are means of five (R-PIA), five (2-Cl-Aden)], three (Pur Rib), three (Ad-N1-Ox)] and two (Ino) experiments, respectively. S.E.M. values were $\pm$ < 15%.

Figure 10

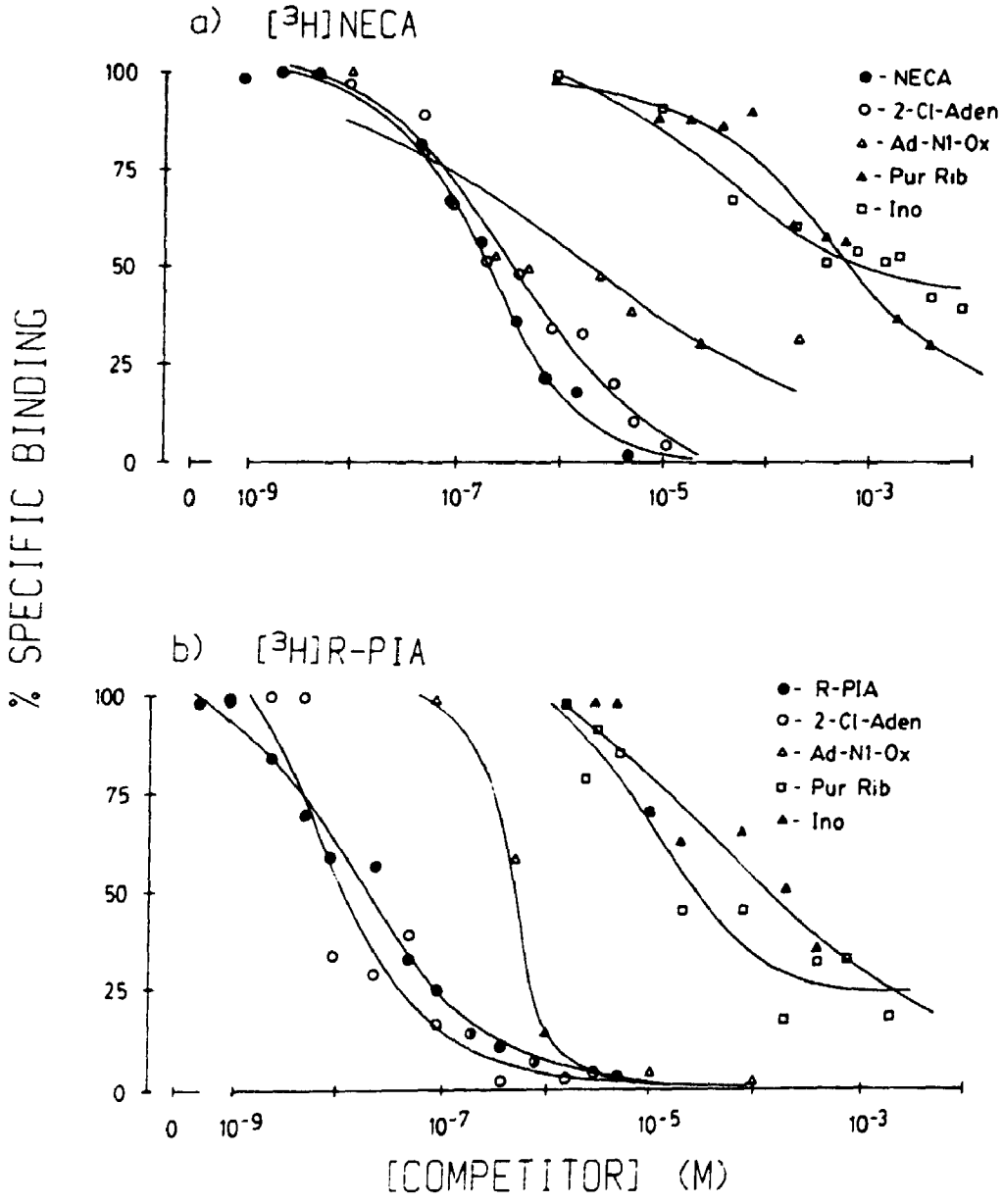

a) [3H]NECA
100
75
50
25
0
0
10-9
10-7
10-5
10-3
● - NECA
○ - 2-Cl-Aden
△ - Ad-N1-Ox
▲ - Pur Rib
□ - Ino
% SPECIFIC BINDING
b) [3H]R-PIA
100
75
50
25
0
0
10-9
10-7
10-5
10-3
● - R-PIA
○ - 2-Cl-Aden
△ - Ad-N1-Ox
□ - Pur Rib
▲ - Ino
[COMPETITOR] (M)

Fig. 11. Displacement curves showing specific binding of [^{3}H]-R-PIA and [^{3}H]-NECA (2.0×10^{-8} M) in the presence of theophylline. The maximum displacement of each radioligand by its unlabeled counterpart was taken as 100% specific binding for that ligand. Displacement by theophylline was related to these values. Values are means of three (R-PIA) and four (NECA) experiments respectively. S.E.M. values were $\pm < 10\%$.

Figure 11

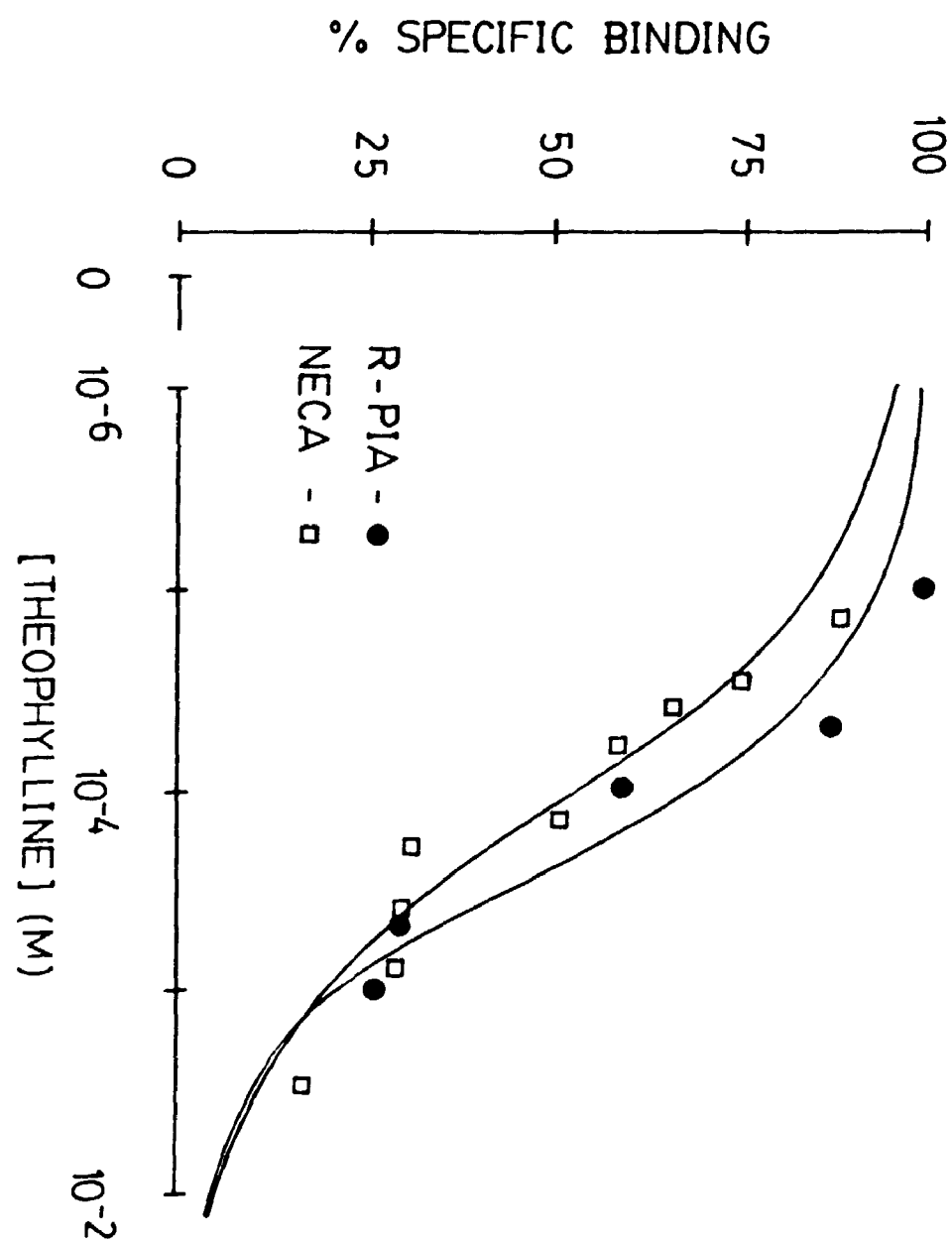

% SPECIFIC BINDING
0
25
50
75
100
0
10-6
10-4
10-2
[THEOPHYLLINE] (M)
R-PIA - ●
NECA - □

Fig. 12. Correlations between the IC_{50} values obtained for various adenosine analogs as displacers of labeled NECA (a) and R-PIA (b) binding and their efficacies as inhibitors of acetylcholine release at the stimulated ileum preparation. The correlation coefficients are 0.998 for R-PIA (y/x = 1.017) and 0.943 for NECA (y/x = 0.893). Slopes of the least squares regression lines were not significantly different from unity ($P > .05$).

Figure 12

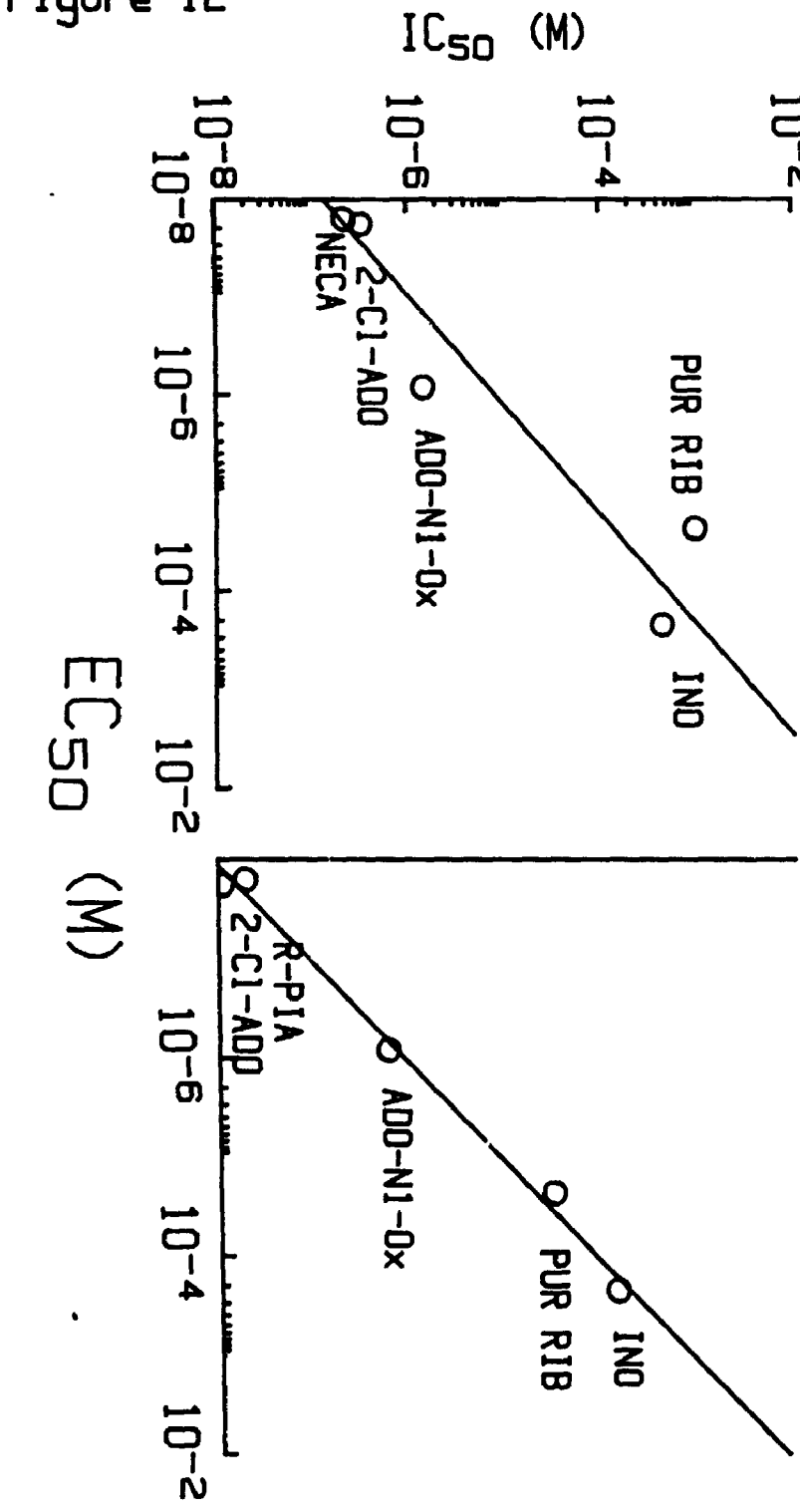

CORRELATION BETWEEN THE BINDING AFFINITIES OF ADO ANALOGS
AND THEIR EFFICACIES AS INHIBITORS OF ACH RELEASE
IC50 (M)
10-2
10-4
10-6
10-8
a)
PUR RIB
IND
ADO-N1-Ox
2-Cl-ADO
NECA
b)
PUR RIB
IND
ADO-N1-Ox
2-Cl-ADO
R-PIA
EC50 (M)
10-8
10-6
10-4
10-2

Fig. 13. Displacement curves showing specific binding of 20 nM [^{3}H]-NECA, [^{3}H]-R-PIA and [^{3}H]-CHA to PV in the presence of various concentrations (M) of their unlabeled counterparts. The maximum displacement of the tritiated ligands by their unlabeled counterparts was taken as 100% specific binding. Values are means of 3-5 experiments and S.E.M. values were $\pm$ < 10%.

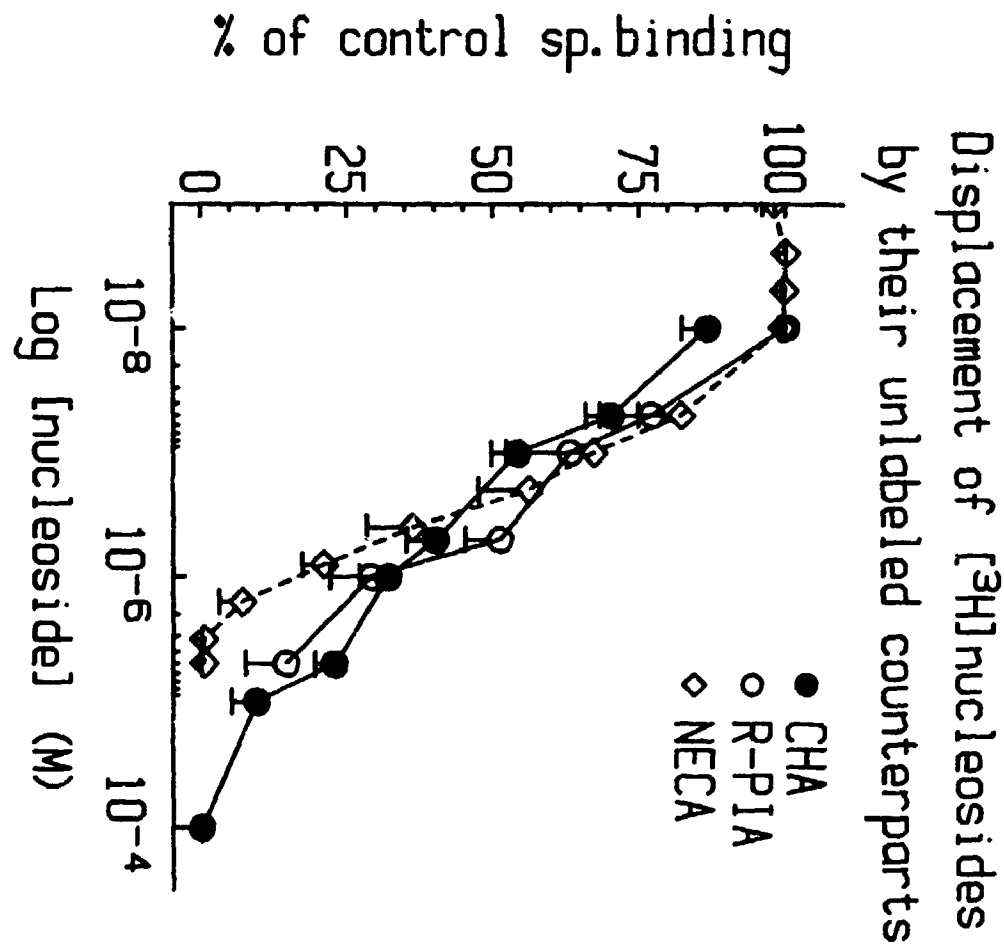

Displacement of [3H]nucleosides
by their unlabeled counterparts
% of control sp. binding
0
25
50
75
100
Log [nucleoside] (M)
10-8
10-6
10-4
● CHA
○ R-PIA
◇ NECA

Figure 13

Fig. 14. a) Displacement curves showing specific binding of [^{3}H]-CHA to PV in the presence of various concentrations of either unlabeled CHA or unlabeled NECA. The maximum displacement of tritiated CHA by its unlabeled counterpart was taken as 100% specific binding. Values are means of 5 experiments and S.E.M. values were $\pm$ < 10%.

b) Displacement curves showing specific binding of [^{3}H]-NECA to PV in the presence of various concentrations of unlabeled NECA, CHA, RPIA, RPBA and CPA. The maximum displacement of tritiated NECA by its unlabeled counterpart was taken as 100% specific binding. Values are means of 3 (CHA and R-FIA), 2 (RPBA, CPA) or 5 (NECA) experiments and S.E.M. values were $\pm$ < 10%. The PV was incubated with nucleoside analogs for 12 minutes.

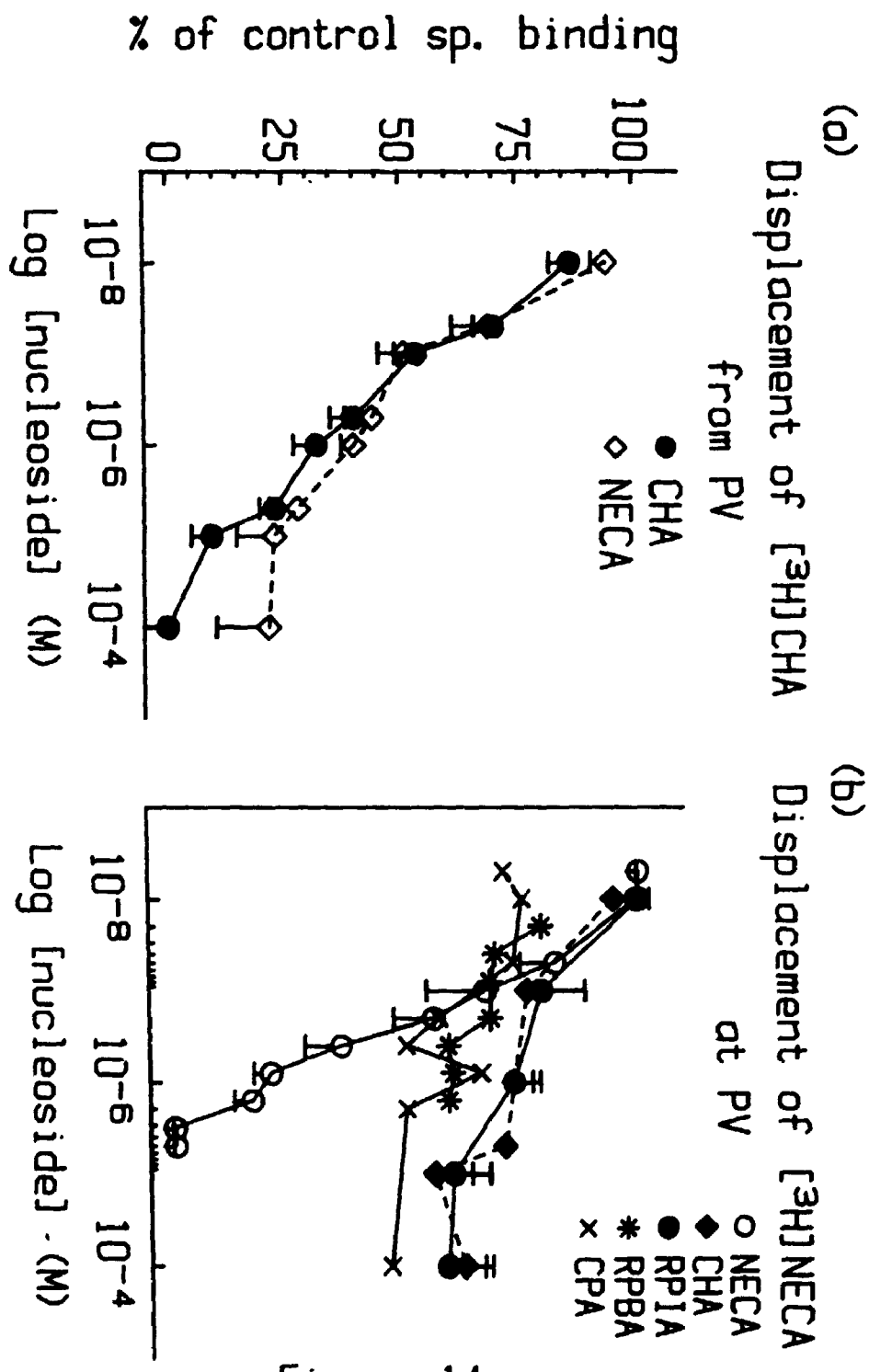

(a)
Displacement of [3H]CHA
from PV
● CHA
◇ NECA
% of control sp. binding
0
25
50
75
100
10-8
10-6
10-4
Log [nucleoside] (M)
(b)
Displacement of [3H]NECA
at PV
○ NECA
◆ CHA
● RPIA
* RPBA
× CPA
10-8
10-6
10-4
Log [nucleoside] (M)

Figure 14

Fig. 15. Displacement curves showing specific binding of [^{3}H]-NECA and [^{3}H]-CHA to PV in the presence of various concentrations of unlabeled DPSPX. The maximum displacement of each labeled ligand by its unlabeled counterpart was taken as 100% specific binding for that ligand. Data shown represent a typical experiment which was repeated twice more with similar results. The PV was incubated with nucleoside analogs for 12 minutes.

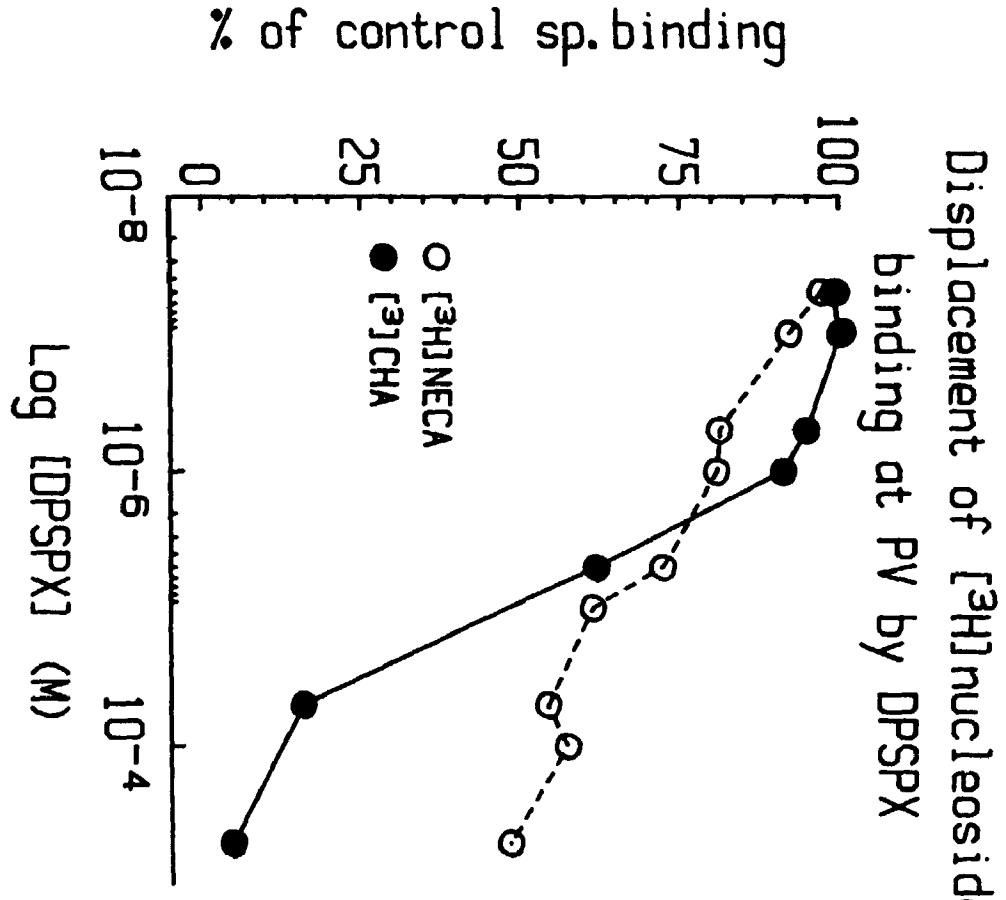
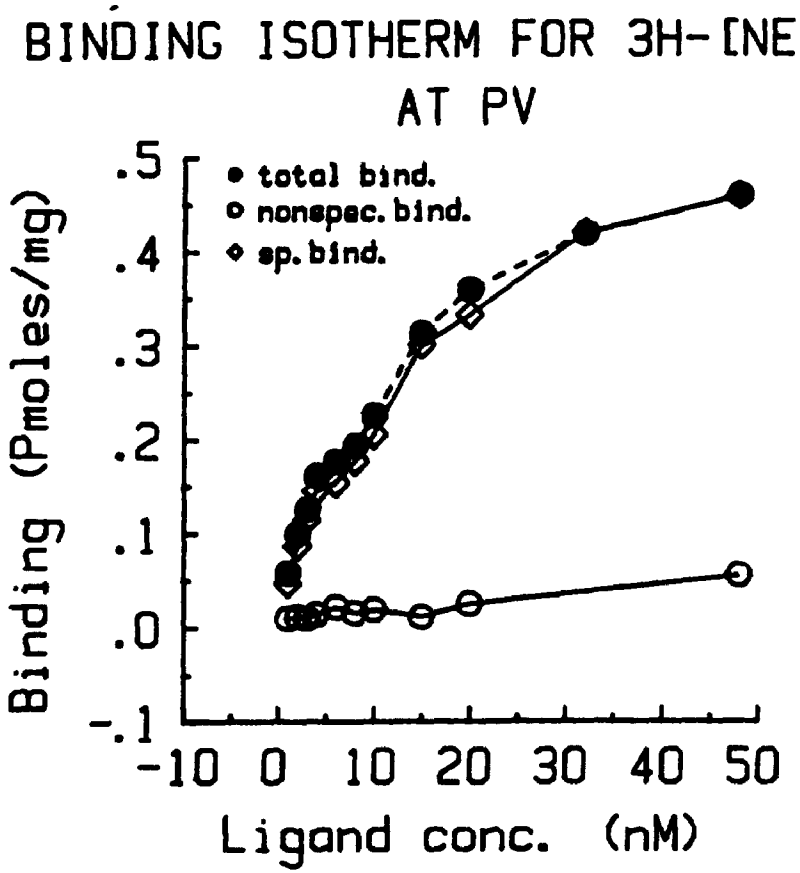

% of control sp.binding
Displacement of [3H]nucleoside
binding at PV by DPSPX
0
25
50
75
100
10-8
10-6
10-4
Log [DPSPX] (M)
O [3H]NECA
● [3]CHA

Figure 15

Fig. 16. Saturation binding isotherms for labeled nucleoside ligands obtained from guinea pig ileal PV. Typical saturation binding curves for a) [^{3}H]-NECA and [^{3}H]-CHA, showing the total, nonspecific and specific binding for these radioligands. The curves were constructed by joining the points. The PV was equilibrated with [^{3}H]-NECA for 1.0h and with [^{3}H]-CHA for 2.0h. at 22 C. The volume of the incubation mixture was 300 ul and approximately 100 ug tissue was used in eachincubation. The experiments were repeated twice more with similar results.

a.


BINDING ISOTHERM FOR 3H-[NECA]
AT PV
Binding (Pmoles/mg)
.5
.4
.3
.2
.1
.0
-.1
• total bind.
o nonspec. bind.
◇ sp. bind.
-10 0 10 20 30 40 50
Ligand conc. (nM)

b.

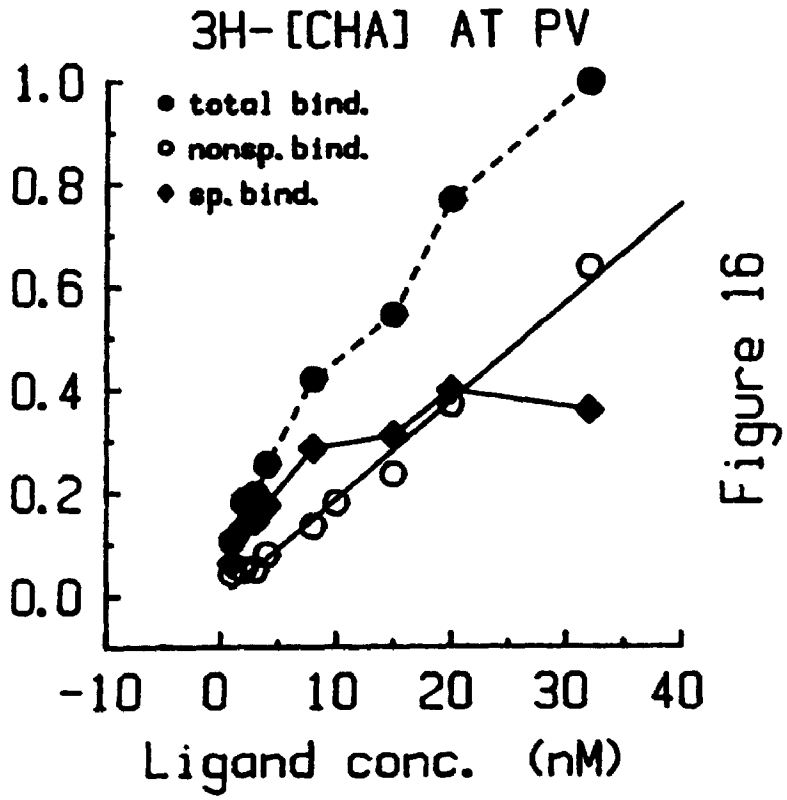

3H-[CHA] AT PV
1.0
0.8
0.6
0.4
0.2
0.0
• total bind.
o nonsp. bind.
◆ sp. bind.
-10 0 10 20 30 40
Ligand conc. (nM)

Figure 16

Fig. 17. Representative Scatchard plots obtained for the binding of a)[^{3}H]-NECA and b) [^{3}H]-CHA at PV. The lines through the points are the least squares regression lines. Similar results were obtained in two additional experiments (see fig. 16 for details).

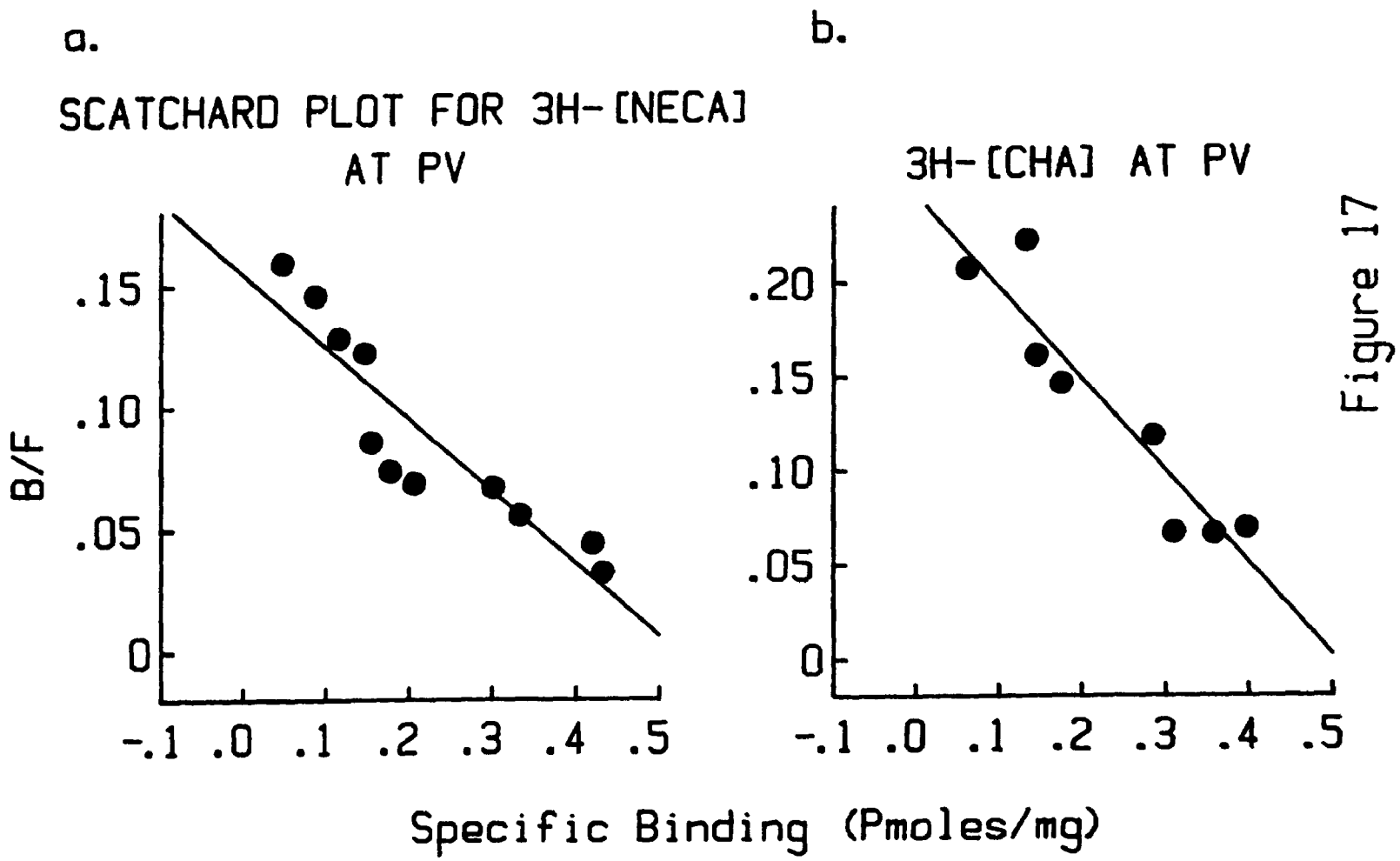

a.
SCATCHARD PLOT FOR 3H-[NECA]
AT PV
B/F
.15
.10
.05
0
-.1 .0 .1 .2 .3 .4 .5
b.
3H-[CHA] AT PV
.20
.15
.10
.05
0
-.1 .0 .1 .2 .3 .4 .5
Specific Binding (Pmoles/mg)

Figure 17

Fig. 18. Saturation binding isotherms for labeled nucleoside ligands obtained at the MITF. Typical saturation binding curves for a) [^{3}H]-NECA and b) [^{3}H]-CHA, showing the total, nonspecific and specific binding for these radioligands. The curves were constructed by joining the points. The MITF was equilibrated with [^{3}H]-NECA for 1.0h and with [^{3}H]-CHA for 2.0h. at 22 C. The volume of the incubation mixture was 300 ul and approximately 300 ug tissue was used in each incubation. The experiments were repeated twice more with similar results.

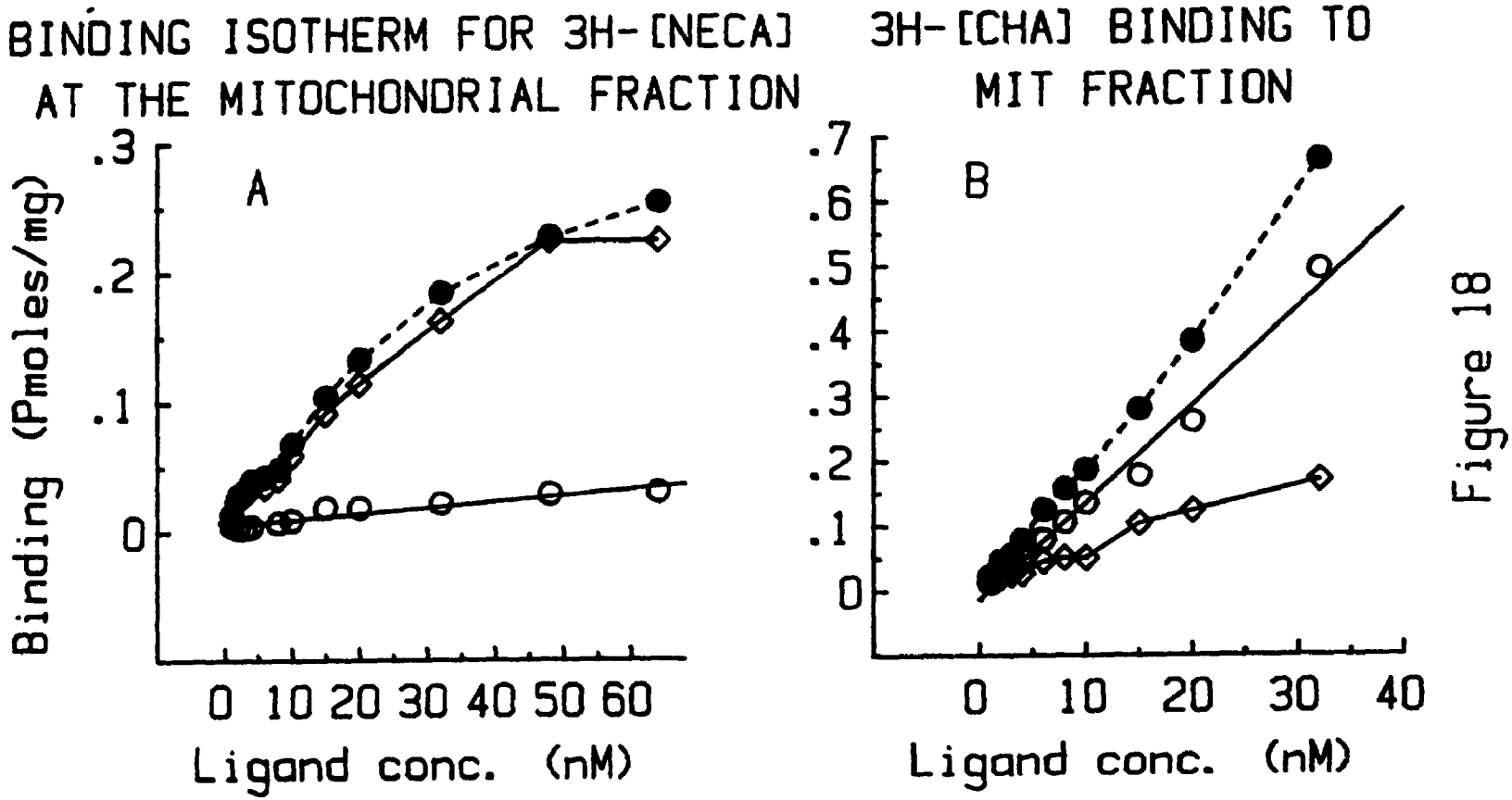

BINDING ISOTHERM FOR 3H-[NECA] AT THE MITOCHONDRIAL FRACTION
Binding (Pmoles/mg)
.3
.2
.1
0
A
0 10 20 30 40 50 60
Ligand conc. (nM)
3H-[CHA] BINDING TO MIT FRACTION
.7
.6
.5
.4
.3
.2
.1
0
B
0 10 20 30 40
Ligand conc. (nM)

Figure 18

Fig. 19. Representative Scatchard plots obtained for the binding of a)[^{3}H]-NECA and b) [^{3}H]-CHA at MITF. The lines through the points are the least squares regression lines. Similar results were obtained in two additional experiments (see fig. 18 for details).

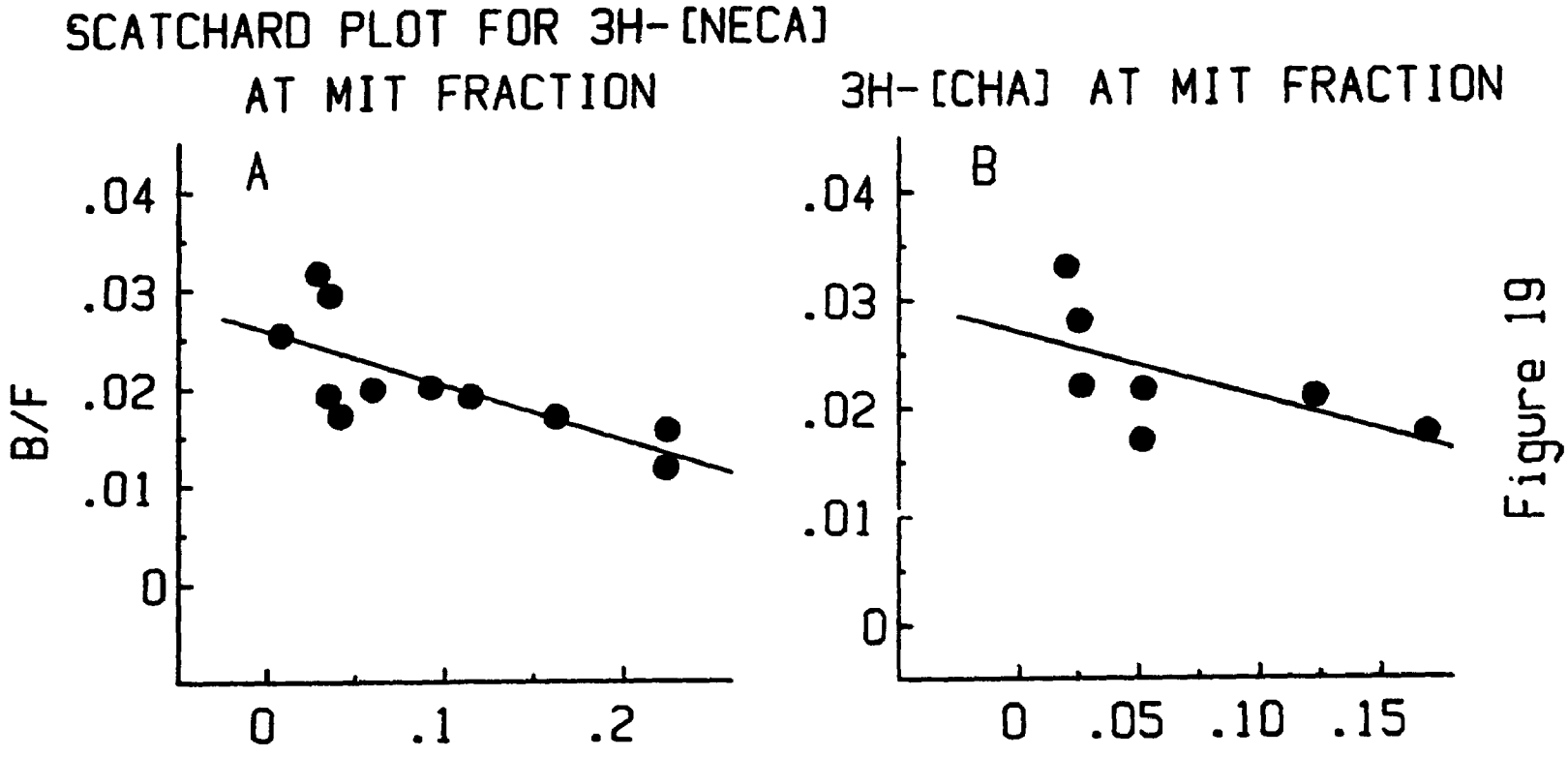

SCATCHARD PLOT FOR 3H-[NECA]
AT MIT FRACTION
3H-[CHA] AT MIT FRACTION
A
B
B/F
.04
.03
.02
.01
0
0
.1
.2
.05
.10
.15

Figure 19

Fig. 20. Comparison of the RIA dose-inhibition curve of SPLI in PV to standard curves produced by synthetic SP (see text for details). The mean ± S.E.M. (n=4) of SPLI content is given at varying dilution points.

Figure 20

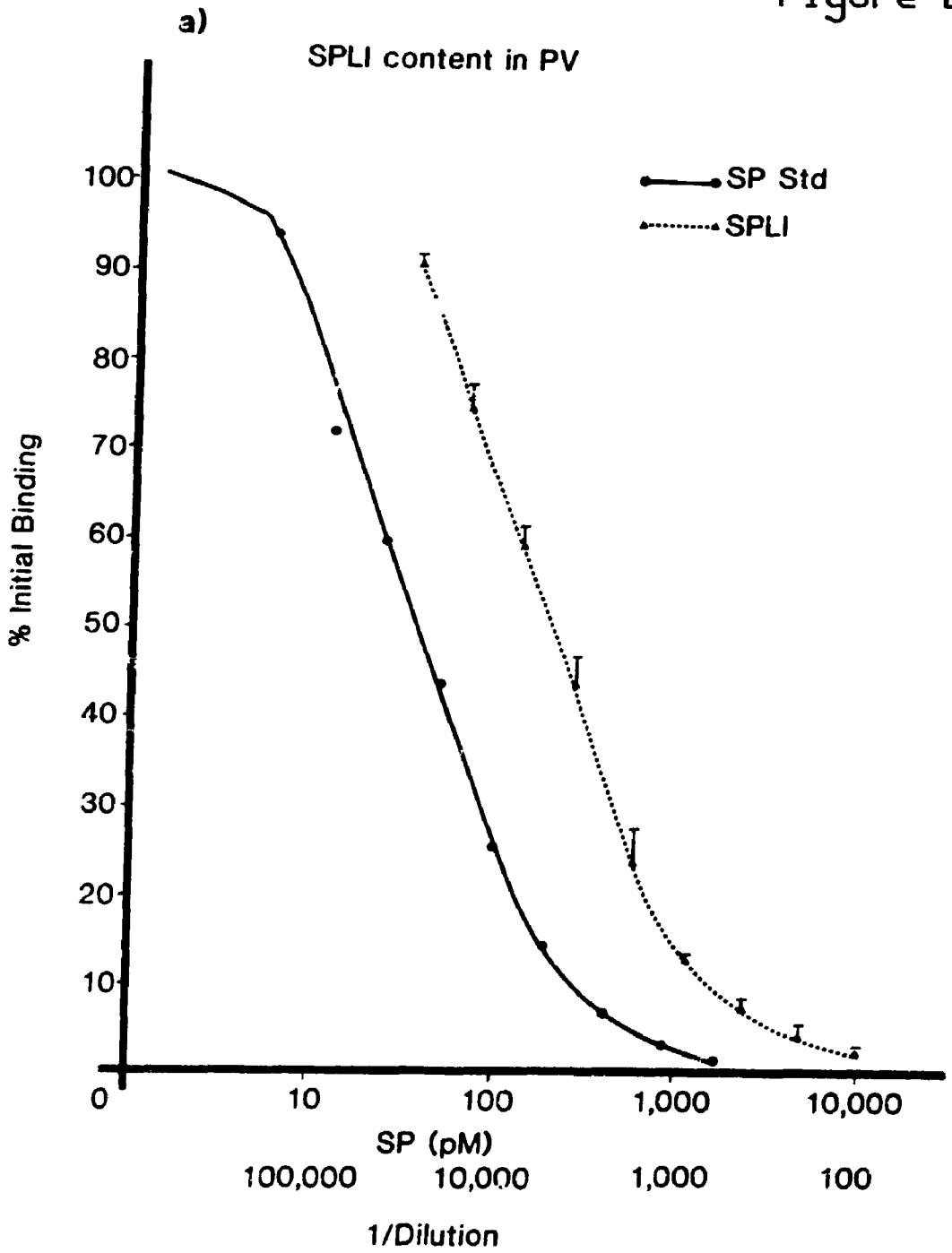

a)
SPLI content in PV
SP Std
SPLI
% Initial Binding
100
90
80
70
60
50
40
30
20
10
0
10
100
1,000
10,000
SP (pM)
100,000
10,000
1,000
100
1/Dilution

Fig. 21. Comparison of the RIA dose-inhibition curve of GAL-LI in PV to standard curves produced by synthetic GAL (see text for details). The mean ± S.E.M. (n=4) of GAL-LI content is given at varying dilution points.

Figure 21

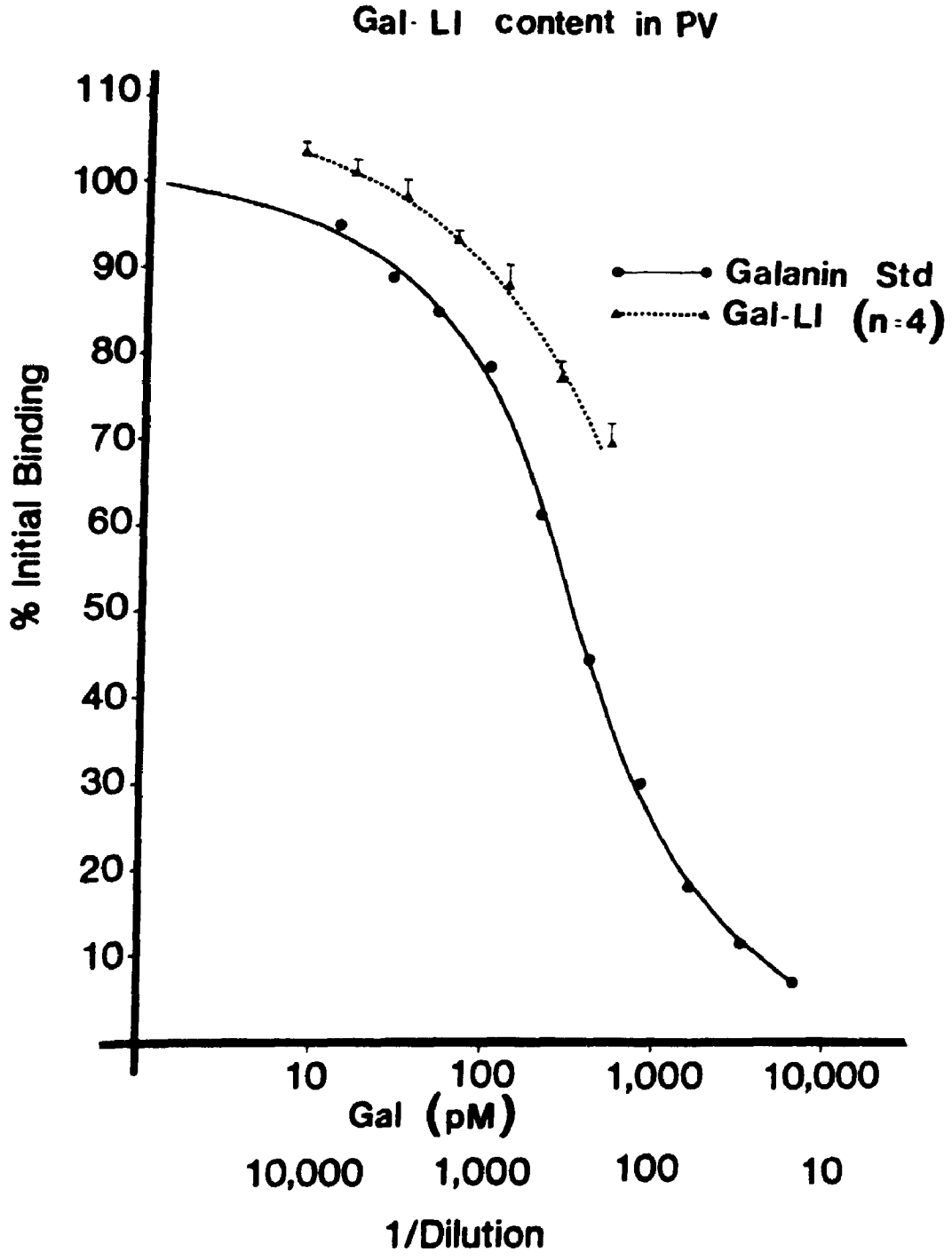

Gal- LI content in PV
110
100
90
80
70
60
50
40
30
20
10
% Initial Binding
Galanin Std
Gal-LI (n=4)
10
100
1,000
10,000
Gal (pM)
10,000
1,000
100
10
1/Dilution

Fig. 22. Comparison of the RIA dose-inhibition curve of VIP-LI in PV to standard curves produced by synthetic VIP (see text for details). The mean ± S.E.M. (n=4) of VIP-LI content is given at varying dilution points.

Figure 22

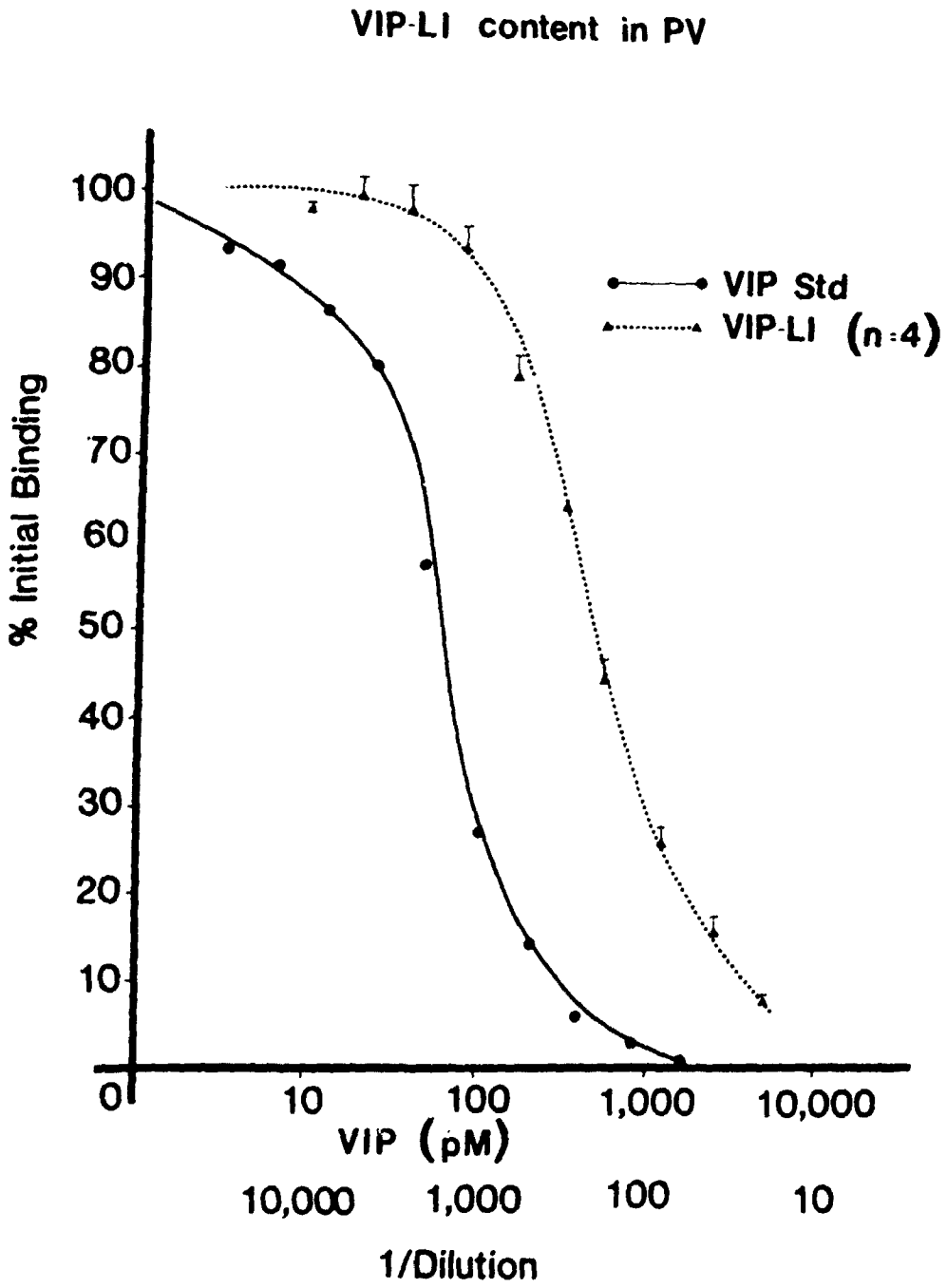

VIP-LI content in PV
100
90
80
70
60
50
40
30
20
10
0
% Initial Binding
VIP Std
VIP-LI (n=4)
10
100
1,000
10,000
VIP (pM)
10,000
1,000
100
10
1/Dilution

Fig. 23. Comparison of the RIA dose-inhibition curve of GRP-LI in PV to standard curves produced by synthetic GRP (see text for details). The mean ± S.E.M. (n=4) of GRP-LI content is given at varying dilution points.

Figure 23

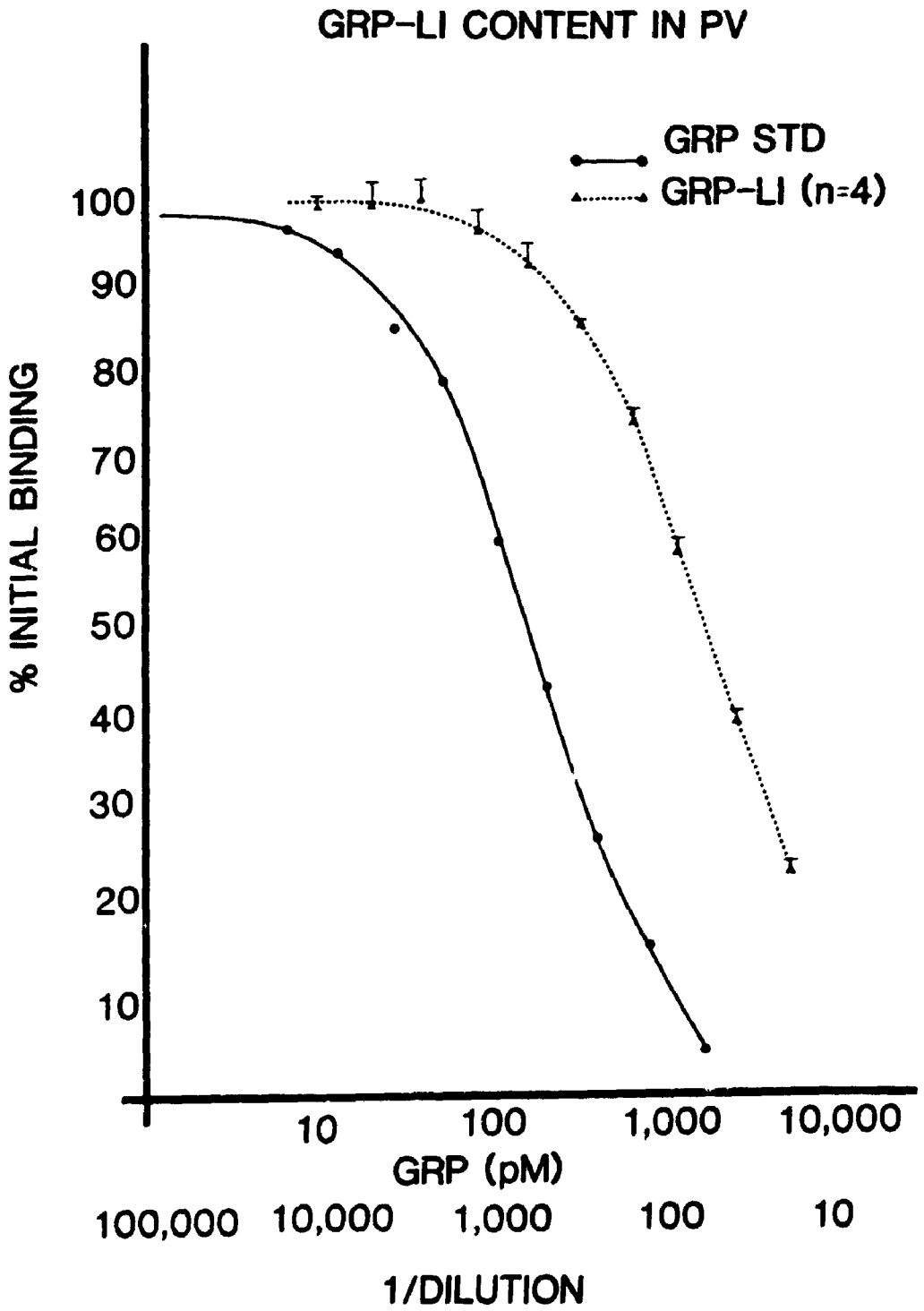

GRP-LI CONTENT IN PV
GRP STD
GRP-LI (n=4)
% INITIAL BINDING
100
90
80
70
60
50
40
30
20
10
10
100
1,000
10,000
GRP (pM)
100,000
10,000
1,000
100
10
1/DILUTION

Fig. 24. Comparison of the RIA dose-inhibition curve of Leu-Enk-LI in PV to standard curves produced by synthetic Leu-Enk-LI (see text for details). The mean ± S.E.M. (n=4) of Leu-Enk-LI content is given at varying dilution points.

Figure 24

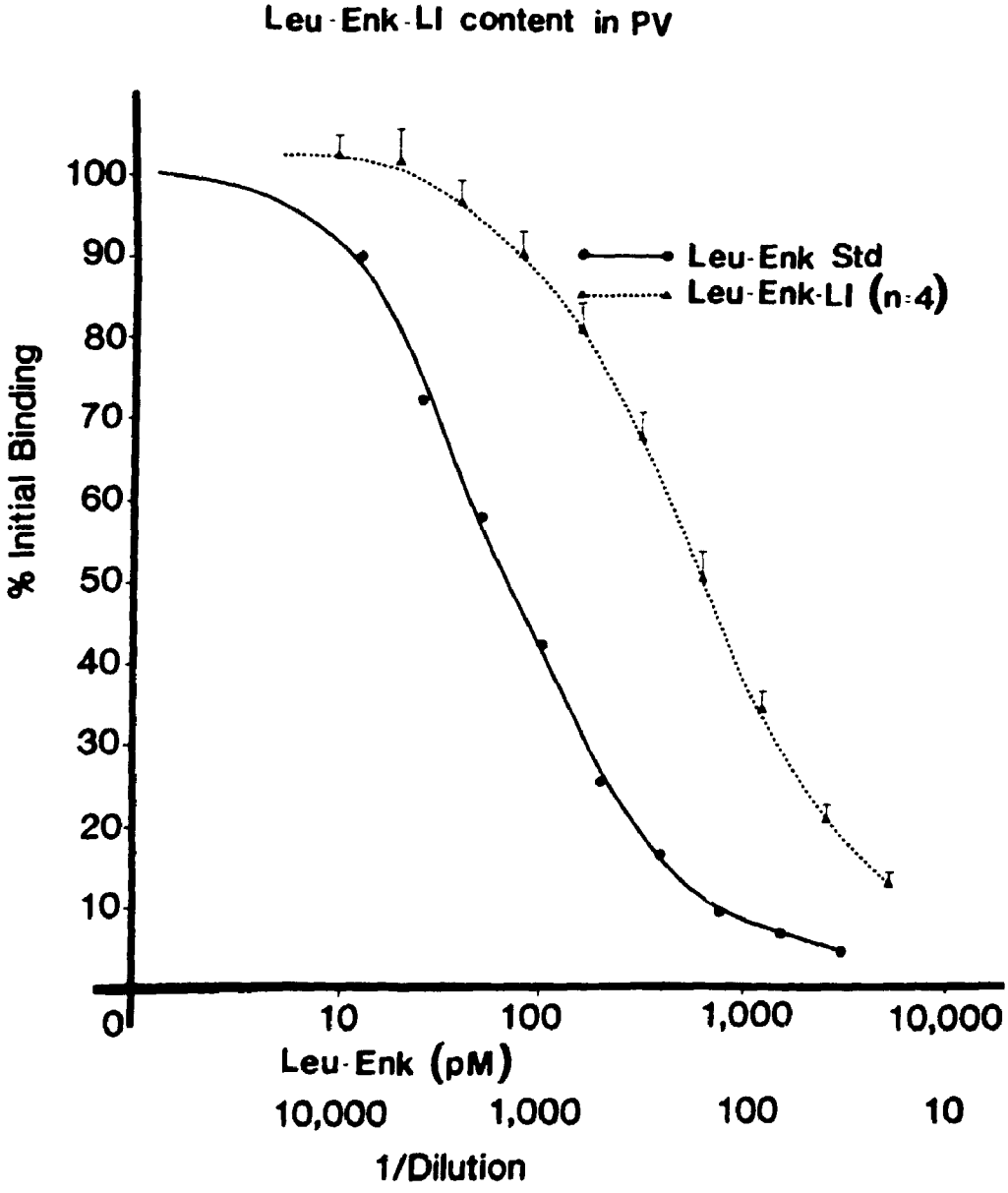

Leu-Enk-LI content in PV
% Initial Binding
100
90
80
70
60
50
40
30
20
10
0
Leu-Enk Std
Leu-Enk-LI (n=4)
10
100
1,000
10,000
Leu-Enk (pM)
10,000
1,000
100
10
1/Dilution

Fig. 25. Comparison of the RIA dose-inhibition curve of Met-Enk-LI in PV to standard curves produced by synthetic Met-EnK (see text for details). The mean ± S.E.M. (n=4) of Met-Enk-LI content is given at varying dilution points.

Figure 25

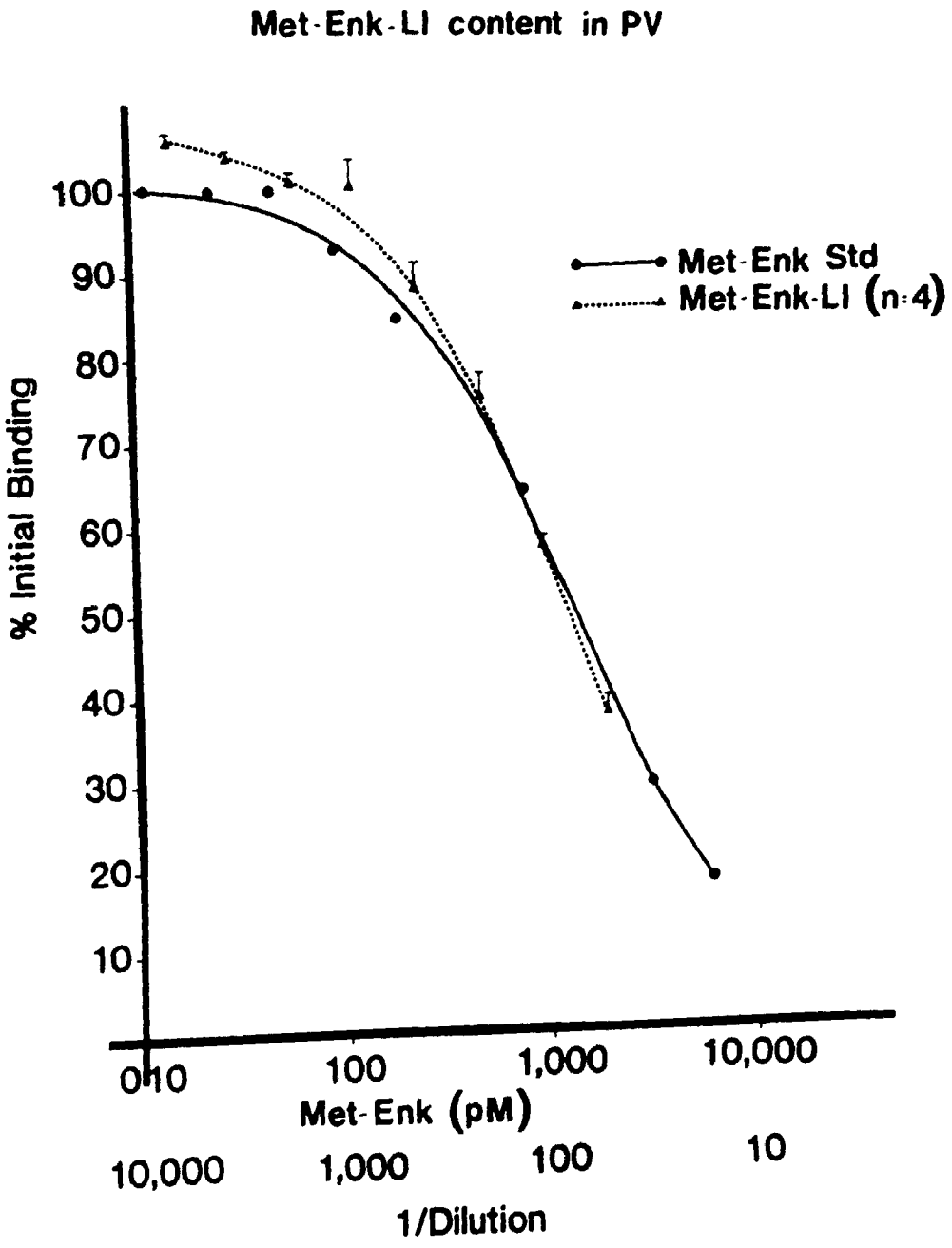

Met-Enk-LI content in PV
% Initial Binding
100
90
80
70
60
50
40
30
20
10
Met-Enk Std
Met-Enk-LI (n=4)
0
10
100
1,000
10,000
Met-Enk (pM)
10,000
1,000
100
10
1/Dilution

Fig. 26. Comparison of the RIA dose-inhibition curve of Enk-LI in PV to standard curves produced by synthetic EnK (see text for details). The mean ± S.E.M. (n=4) of Enk-LI content is given at varying dilution points.

Figure 26

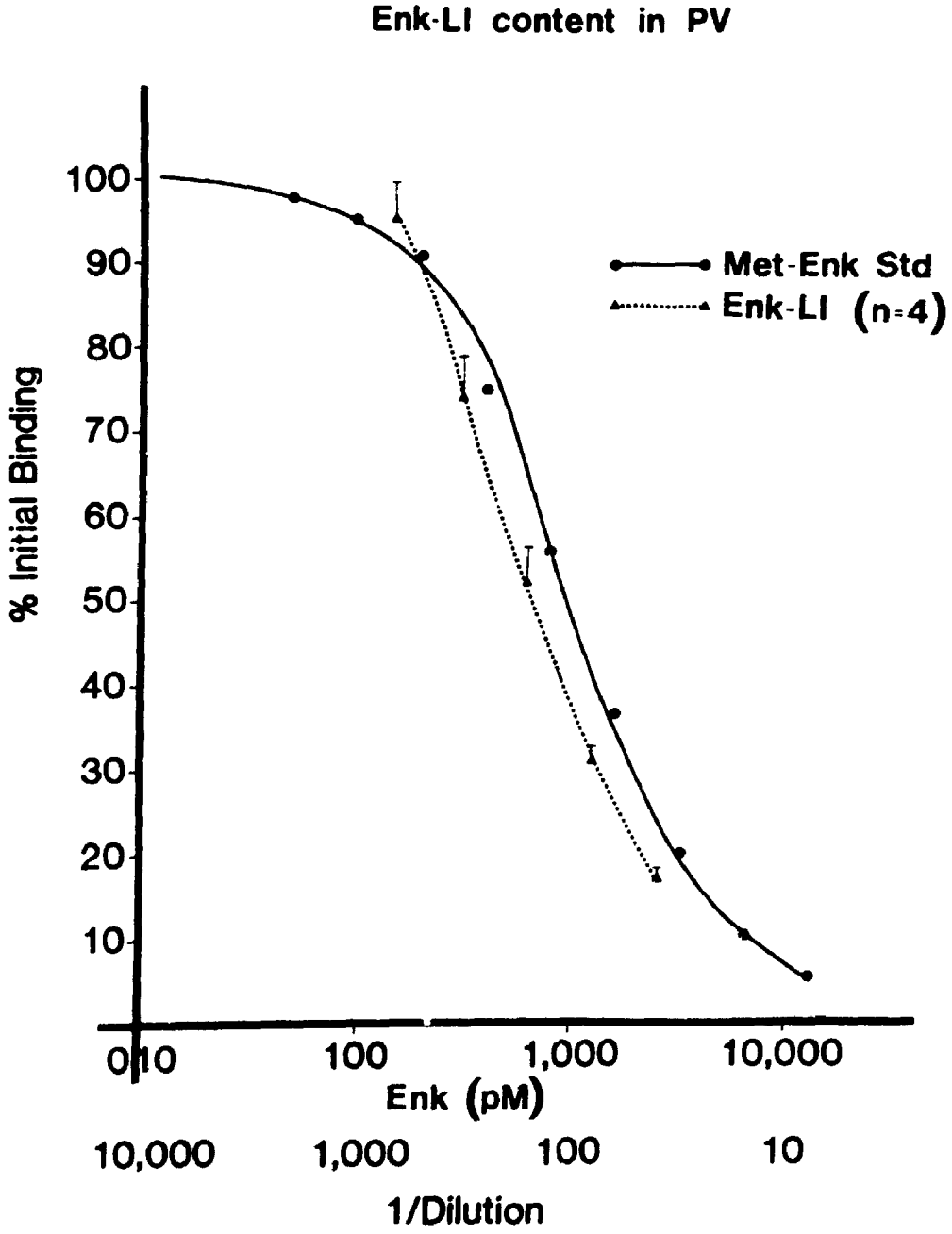

Enk-LI content in PV
100
90
80
70
60
50
40
30
20
10
% Initial Binding
Met-Enk Std
Enk-LI (n=4)
0
10
100
1,000
10,000
Enk (pM)
10,000
1,000
100
10
1/Dilution

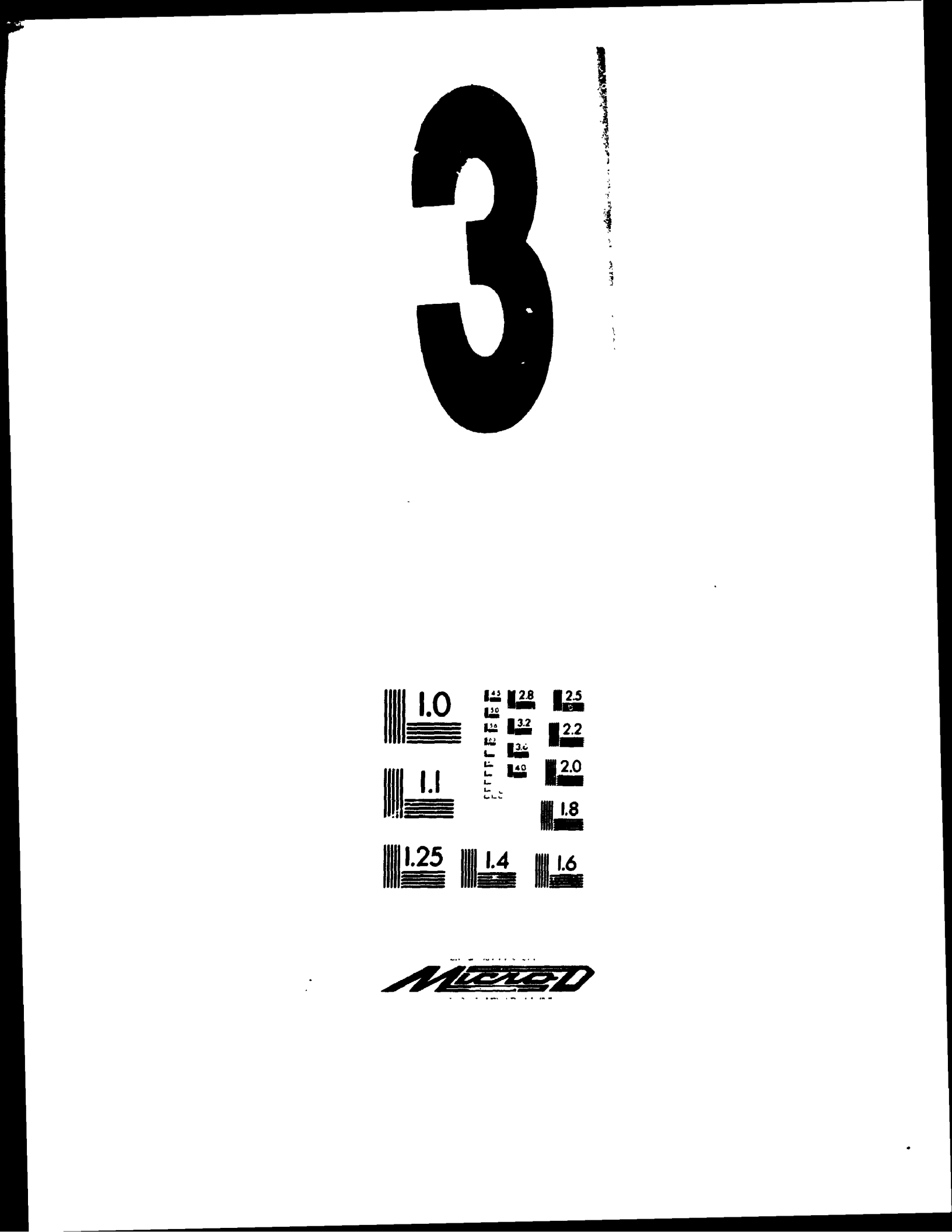
Fig. 27. Comparison of occluded LDH activity in several fractions obtained during the isolation of purified myenteric varicosities from guinea pig ileal-longitudinal muscle strips. The occluded LDH activity was measured in fractions P_2, F_a, F_b and F_c. Fractions F_a, F_b and F_c represent clearly visible bands on continuous sucrose gradients as described in the methods. The sucrose densities at the center of these bands are 0.648 $\pm$ .11M, 1.027 $\pm$ .07M and 1.257 $\pm$ .06M respectively. Each bar represents the mean $\pm$ S.E.M from four separate experiments.

more potent than the S-isomer. R-PIA was about 10 times more potent than S-PIA.

Antagonism of inhibitory effects of nucleosides at the stimulated ileum by xanthine analogs.

Dose-effect curves to several nucleosides were obtained in the absence and presence of incrementing concentrations of theophylline or DPSPX. Schild plots of these data are shown for theophylline in figure 3a to 3d. Linear isoboles with unit slopes ($p>.05$) were obtained for the analogs NECA, R-PIA, CHA and CPA against theophylline and the pA_2 values obtained are listed in table 1.

Schild analysis using DPSPX (see fig 4) showed linear isoboles with unit slope ($p>.05$) for both CHA and MCPA while NECA displayed a curvilinear isobole. The 95% confidence intervals calculated for the dose-ratios showed that, at a DPSPX concentration of 5 uM the values obtained for NECA were significantly different ($p<.05$) from those obtained for either of the A_1 agonists. Further, the values for NECA and CHA at 2 uM were also significantly different ($p<0.05$). Similar analysis of the isoboles at lower antagonist concentrations showed no such significant differences. The deviation of the Schild isobole from unit slope thus commences at an ordinate value (DR-1) approaching 1.0. For comparison purposes, the linear isoboles for CHA and NECA obtained against theophylline are also presented in fig 4. The pA_2 values obtained for several adenosine analogs against DPSPX are shown in Table 1.


3
1.0
1.1
1.25
1.4
1.6
2.8
2.5
3.2
2.2
3.6
2.0
4.0
1.8
MicroD

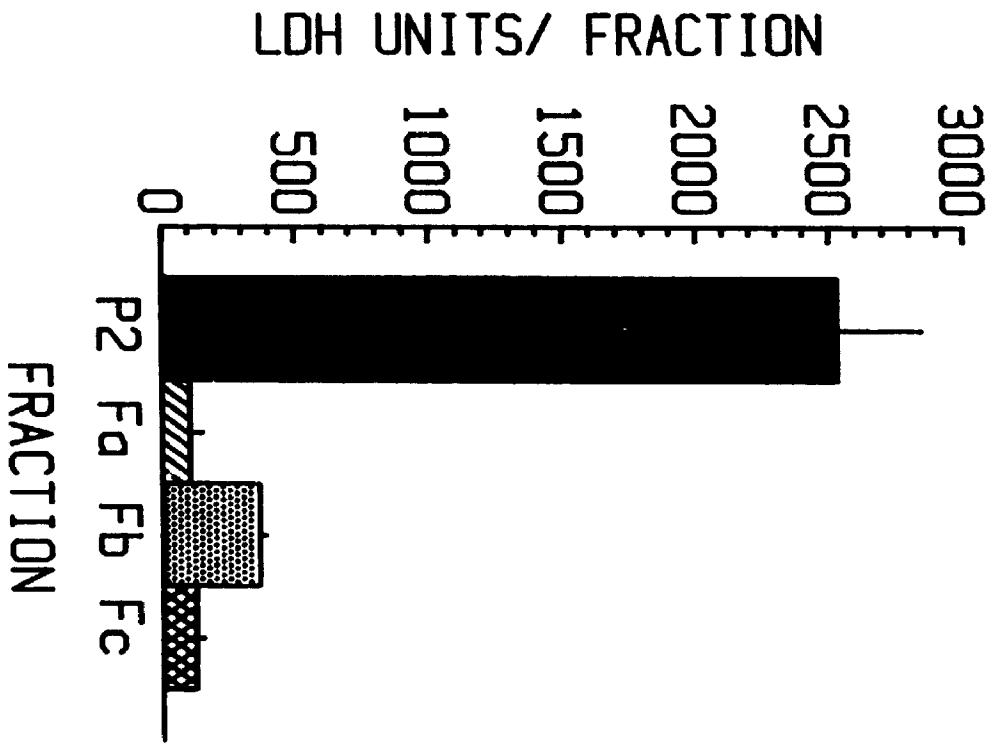

LDH UNITS/ FRACTION
0
500
1000
1500
2000
2500
3000
FRACTION
P2 Fa Fb Fc

Figure 27

Fig. 28. Distribution of peptide-like immunoreactivities in several fractions obtained during the isolation of purified myenteric varicosities from guinea pig ileal longitudinal muscle-myenteric plexus strips. The peptide-like immunoreactivities were measured by specific RIA's. Fractions Fa, Fb and Fc represent clearly visible bands on continuous sucrose gradients. The sucrose densities at the center of these bands are 0.648 $\pm$ .11M, 1.027 $\pm$ .07M and 1.257 $\pm$ .06M respectively. Each bar represents the mean $\pm$ S.E.M from four separate experiments. For measuring the contents of peptide-immunoreactivities, aliquots of each fraction were centrifuged and then boiled for 7 min. to inactivate proteolytic enzymes. The samples were frozen at -70 C until performance of the specific RIA's. a) Distribution of Leu-EnK-LI; b) distribution of Met-Enk-LI; c) distribution of VIP-LI; d) distribution of GRP-LI.

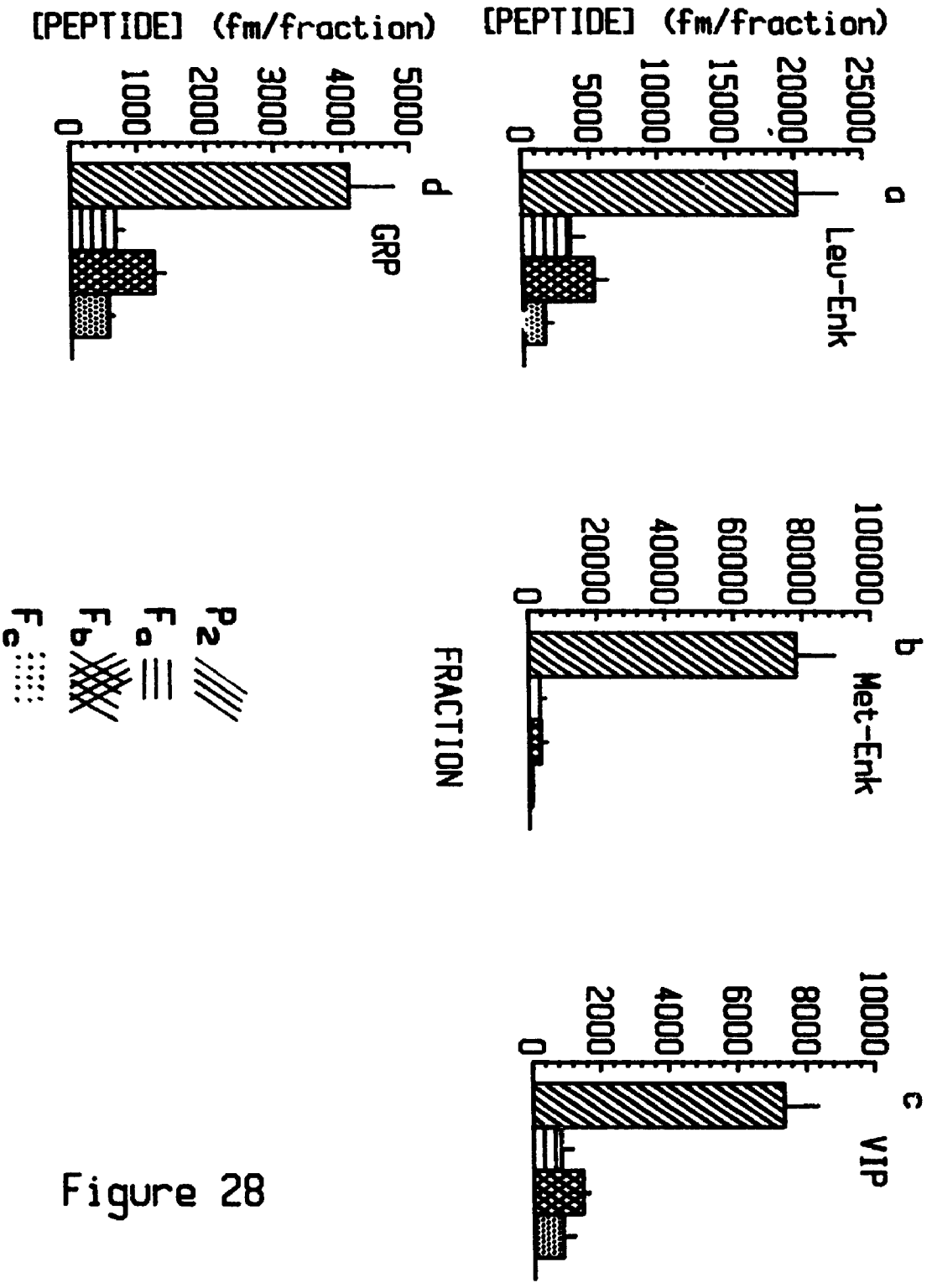

[PEPTIDE] (fm/fraction)
0
5000
10000
15000
20000
25000
a
Leu-Enk
[PEPTIDE] (fm/fraction)
0
1000
2000
3000
4000
5000
d
GRP
FRACTION
0
20000
40000
60000
80000
100000
b
Met-Enk
0
2000
4000
6000
8000
10000
c
VIP
P2
Fa
Fb
Fc

Figure 28

Fig. 29. Histogram, showing te ratio of the content of peptide immunoreactivities in the P_2 fraction to that in the Fb fraction. Ratios for GRP-LI, Leu-EnK-LI (L-E), Met-Enk-LI and VIP-LI are shown. Data were normalized to protein in each fraction and numbers used in the ratio analysis represent the total amount of immunoreactivity from the ileal LMMP of one guinea pig. The ratio of occluded LDH activity is also presented. The values represent the mean $\pm$ the S.E.M. from 4 separate experiments. The ratio obtained for Met-Enk-LI is significantly greater ($p < .001$) than all other ratios presented. For measuring the contents of peptide-immunoreactivities, aliquots of each fraction were centrifuged and then boiled for 7 min. to inactivate proteolytic enzymes. The samples were frozen at -70 C until performance of the specific RIA's.

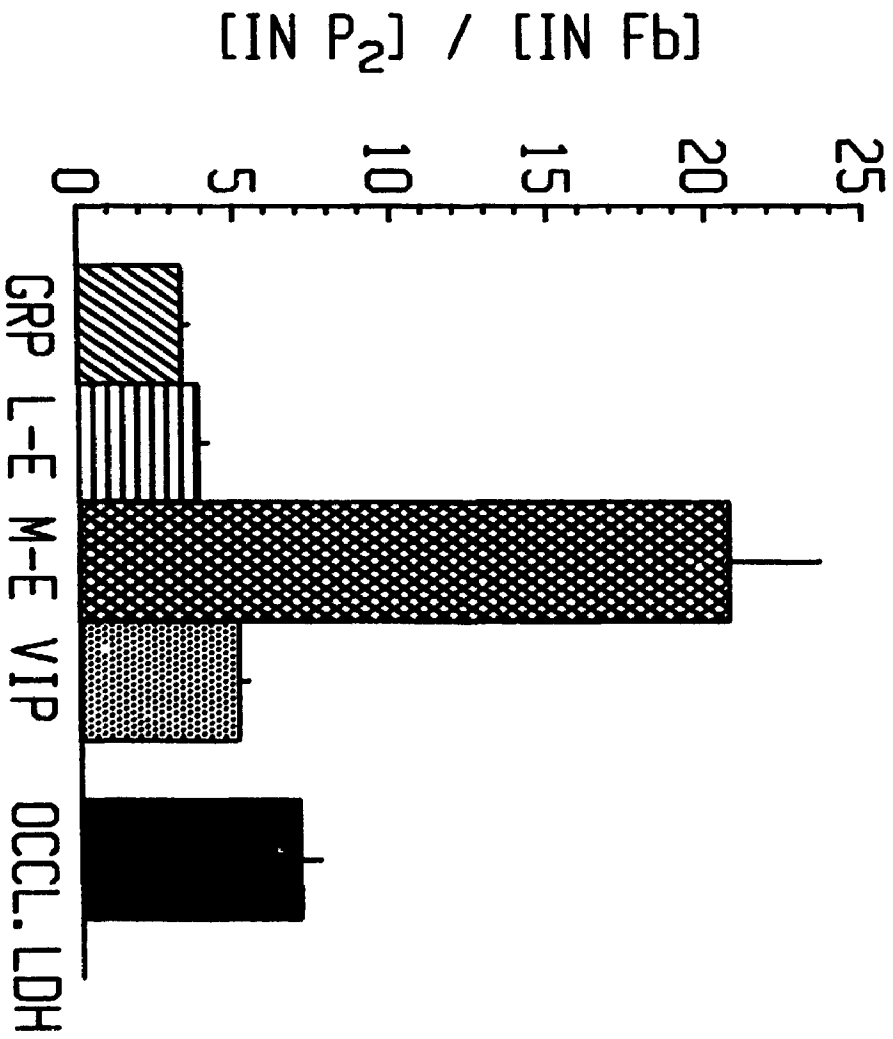


Figure 29

Fig. 30. Photograph of a typical continuous sucrose gradient showing the separation of the P_2 suspension into 3 clear bands at F_a, F_b and F_c. The gradients were formed using the layering method as described in the methods. The gradients were cooled to the ultracentrifuge running temperature (4 C), 1.9 ml of P_2 suspension was layered on top of the sucrose gradient and centrifuged at 150,000 x g (35,000 rpm) for 90 min using the SW41 Ti rotor. The molarity of the sucrose at these bands was obtained from control gradient tubes in which the P_2 was omitted (n=5). Similar band distribution obtained in 7 separate experiments.

Fig 30

F_a 0.648±.11M

F_b 1.027±.07M

F_c 1.257±.06M

Fig. 31. Reverse-phase HPLC characteristics of SPLI in PV preparations. The elution positions of synthetic peptides and their principle oxidized (ox) form(s) are indicated on the chromatograms by arrows and specific labels. The peak amounts of the SPLI eluting near the position of standard SP are given in the brackets as pmol/fraction. Immunoreactive entities are indicated by the solid bars. a) elution under 'steep' gradient elution conditions (see text for details). b) elution under 'shallow'gradient conditions (see text for details). These are representative results from one of four experiments.

Figure 31a

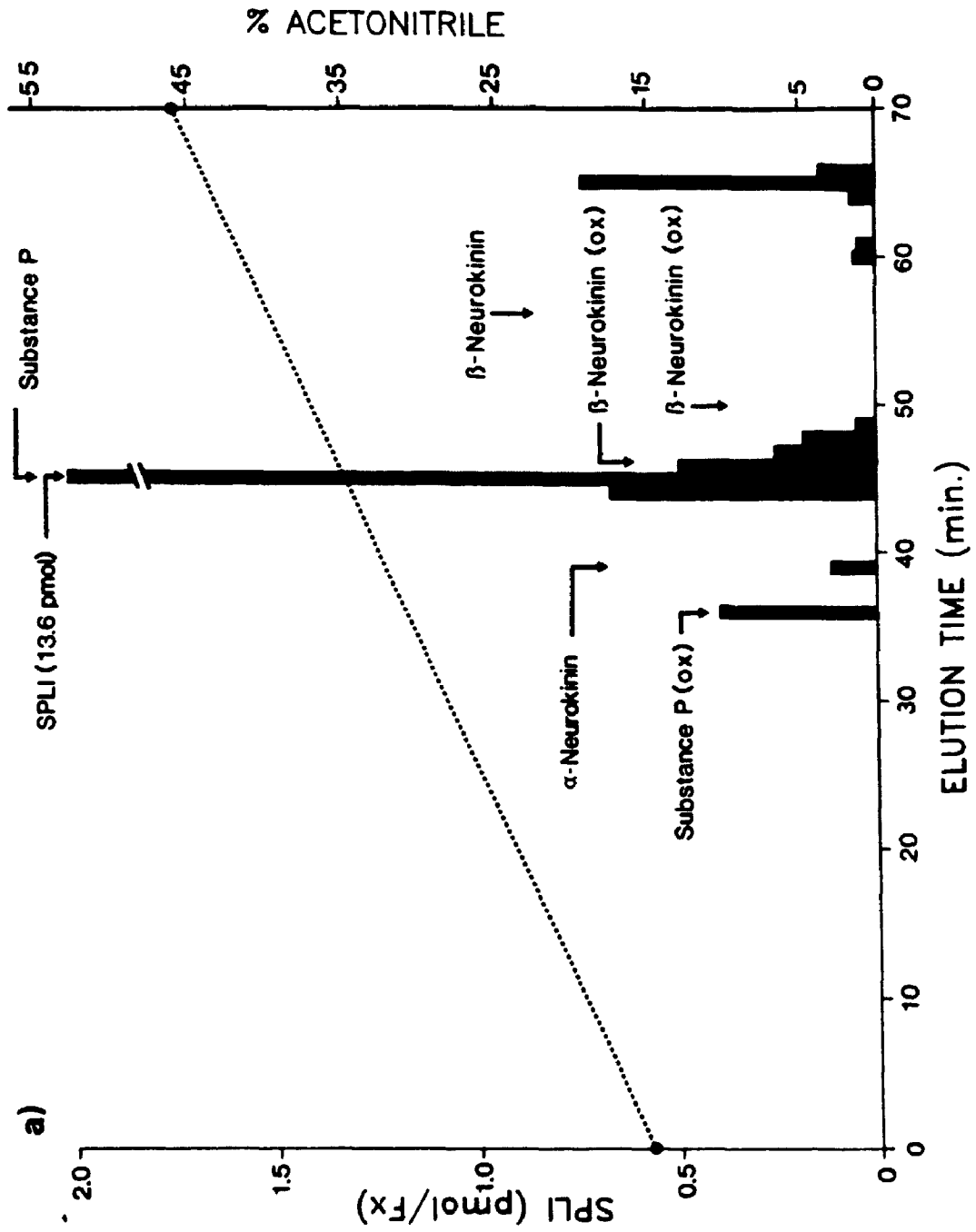

% ACETONITRILE
55
45
35
25
15
5
0
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
Substance P
SPLI (13.6 pmol)
ß-Neurokinin
ß-Neurokinin (ox)
ß-Neurokinin (ox)
α-Neurokinin
Substance P (ox)
a)
2.0
1.5
1.0
0.5
0
SPLI (pmol/Fx)

Figure 31b

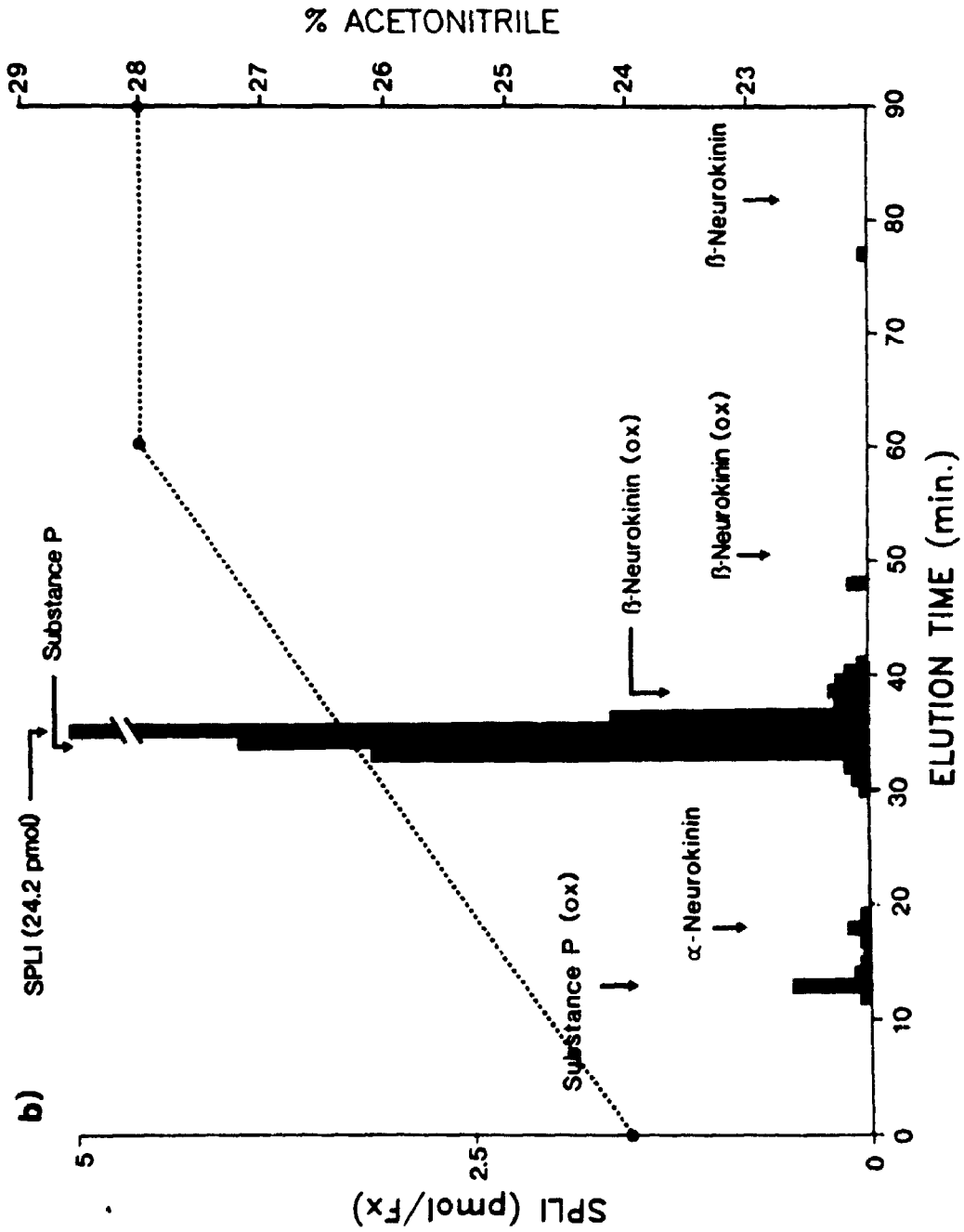

% ACETONITRILE
29
28
27
26
25
24
23
90
80
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
ß-Neurokinin
ß-Neurokinin (ox)
ß-Neurokinin (ox)
Substance P
SPLI (24.2 pmol)
α-Neurokinin
Substance P (ox)
b)
5
2.5
0
SPLI (pmol/Fx)

Fig. 32. Reverse phase HPLC elution patterns of LeuEnkLI and EnkLI in PV preparations as measured by: a) the specific Leu-enkephalin antiserum and b) the antiserum recognizing both enkephalins (see text for details). The PV preparations injected onto the reverse-phase column were eluted with 'steep' gradient elution conditions (see text for details). The elution position for synthetic Leu- and Met-enkephalin are indicated by the arrows. Immunoreactive entities are indicated by the solid bars. These are representative results from one of four experiments.

Figure 32a

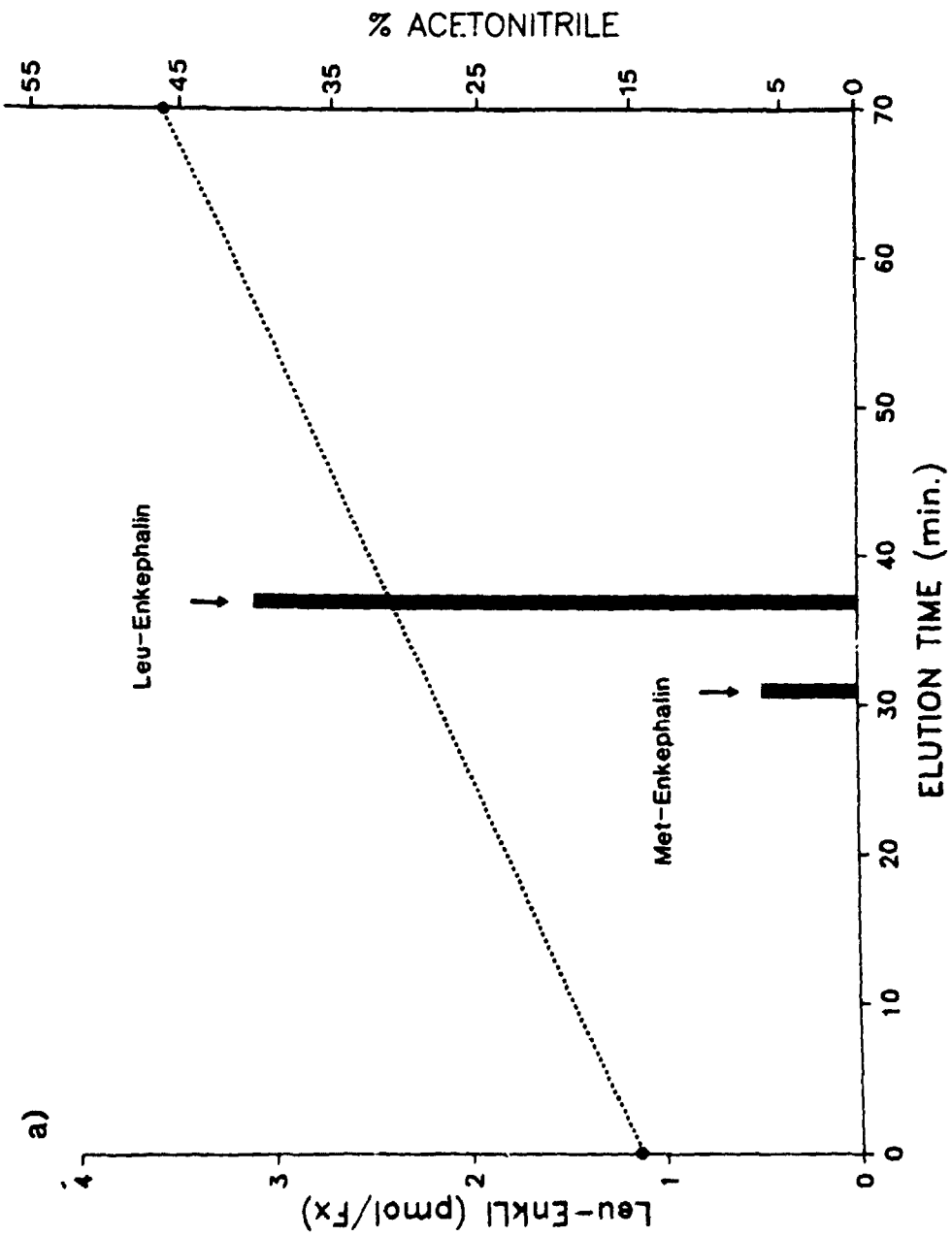

% ACETONITRILE
55
45
35
25
15
5
0
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
Leu-Enkephalin
Met-Enkephalin
a)
4
3
2
1
0
Leu-Enkl (pmol/fx)

Figure 32b

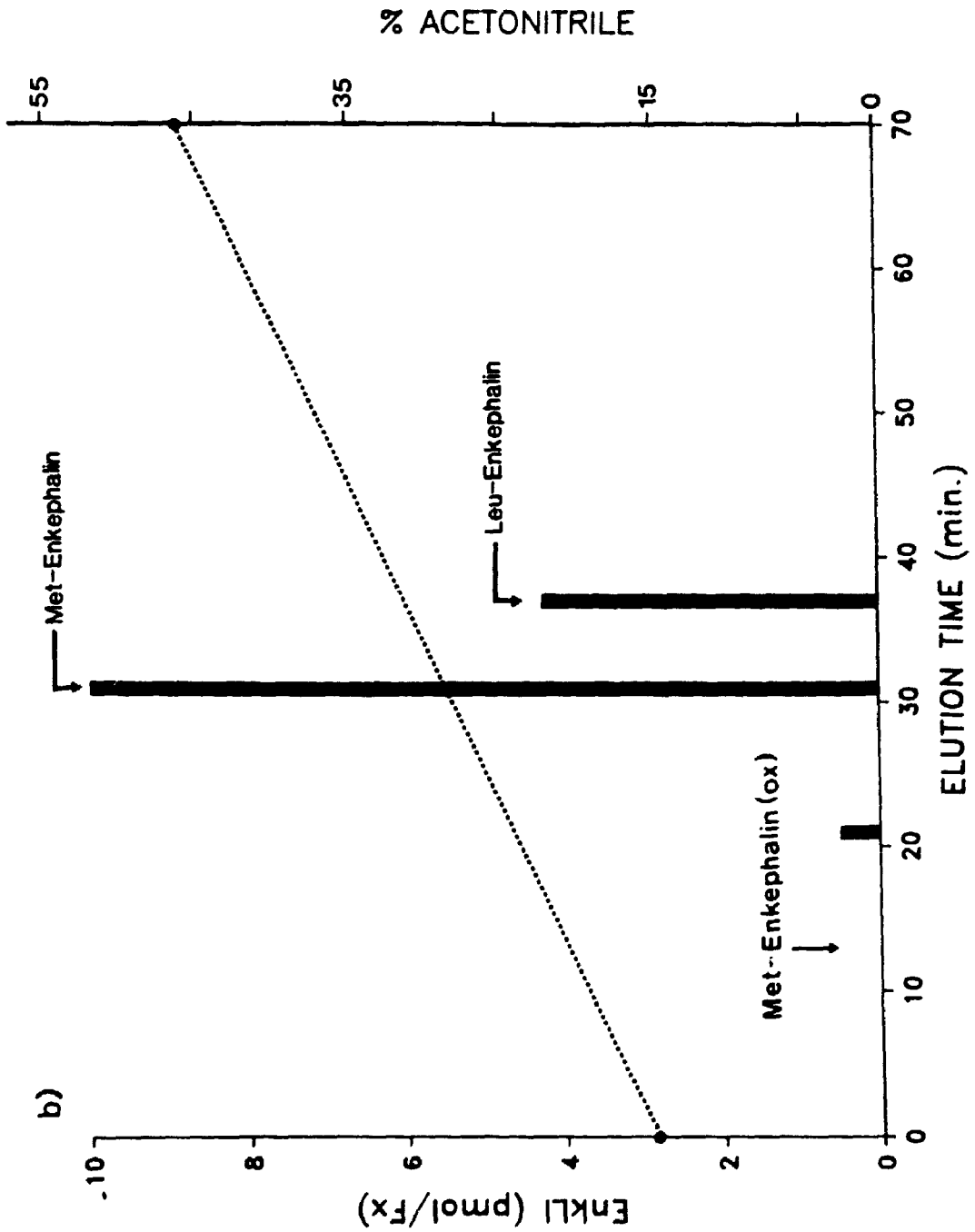

% ACETONITRILE
55
35
15
0
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
Met-Enkephalin
Leu-Enkephalin
Met-Enkephalin(ox)
b)
-10
8
6
4
2
0
EnkLI (pmol/Fx)

Fig. 33. Reverse-phase HPLC of GALLI in PV preparations under 'steep' gradient elution conditions (see text for details). The elution position of synthetic porcine galanin is marked by the arrows and GALLI is represented by the solid bars. These are representative results from one of four experiments.

Figure 33

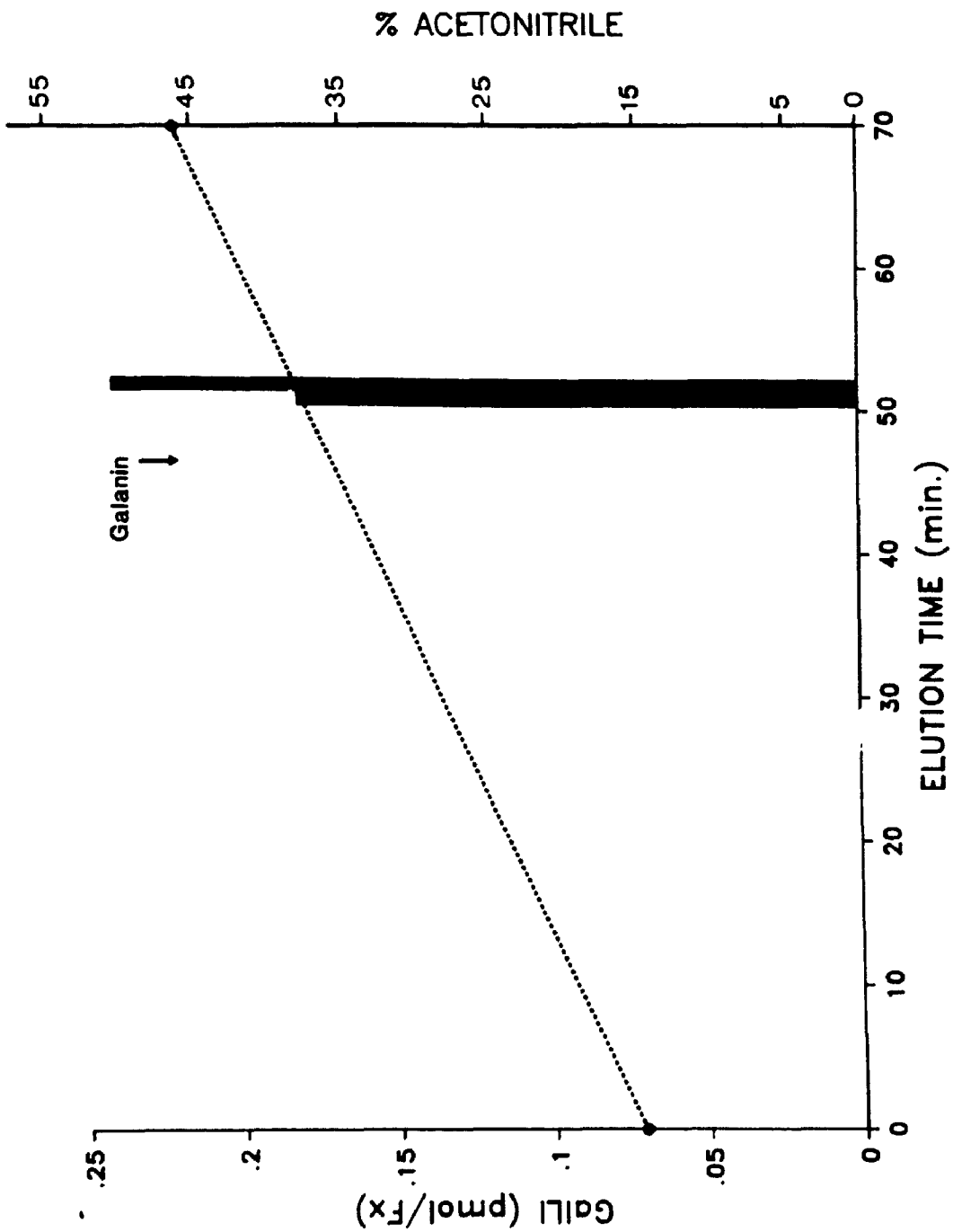

% ACETONITRILE
55
45
35
25
15
5
0
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
Galanin
GalLI (pmol/Fx)
.25
.2
.15
.1
.05
0

Fig. 34. Reverse-phase HPLC characteristics of GRPLI in PV preparations under 'shallow' gradient elution conditions (see text for details). The elution position of synthetic porcine GRP-(1-27), its principle oxidized (ox) form and the elution position of GRP (18-27) are marked by arrows. GRPLI is represented by the solid bars. These are representative results from one of four experiments.

Figure 34

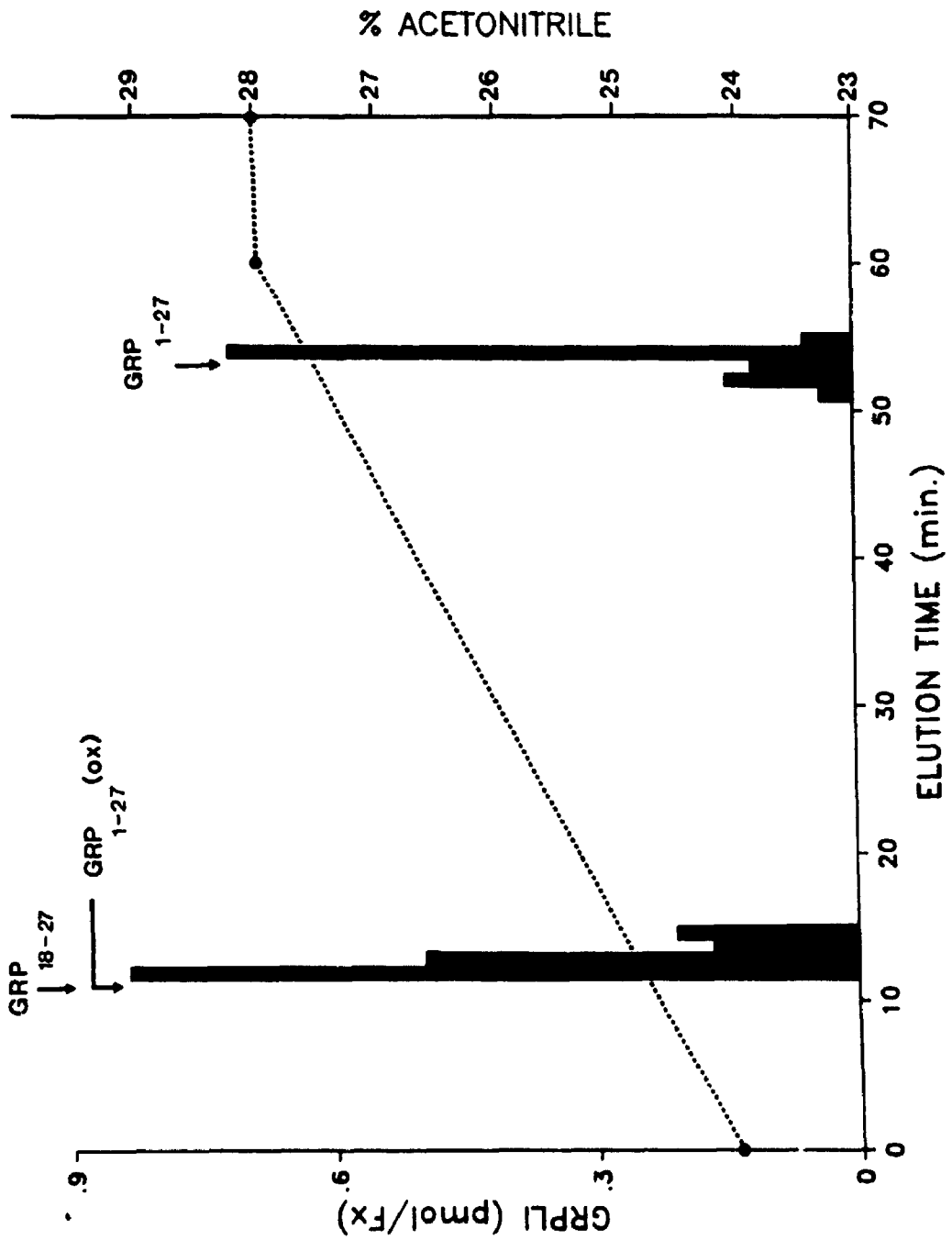

% ACETONITRILE
29
28
27
26
25
24
23
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
GRP 1-27
GRP (ox) 1-27
GRP 18-27
.9
.6
.3
0
GRPLI (pmol/Fx)

Fig. 35. Reverse-phase HPLC characteristics of VIPLI in PV preparations under 'shallow' gradient elution conditions (see text for details). The elution position of synthetic porcine VIP is indicated by the arrow and VIPLI by the solid bars. These are representative results from one of four experiments.

Figure 35

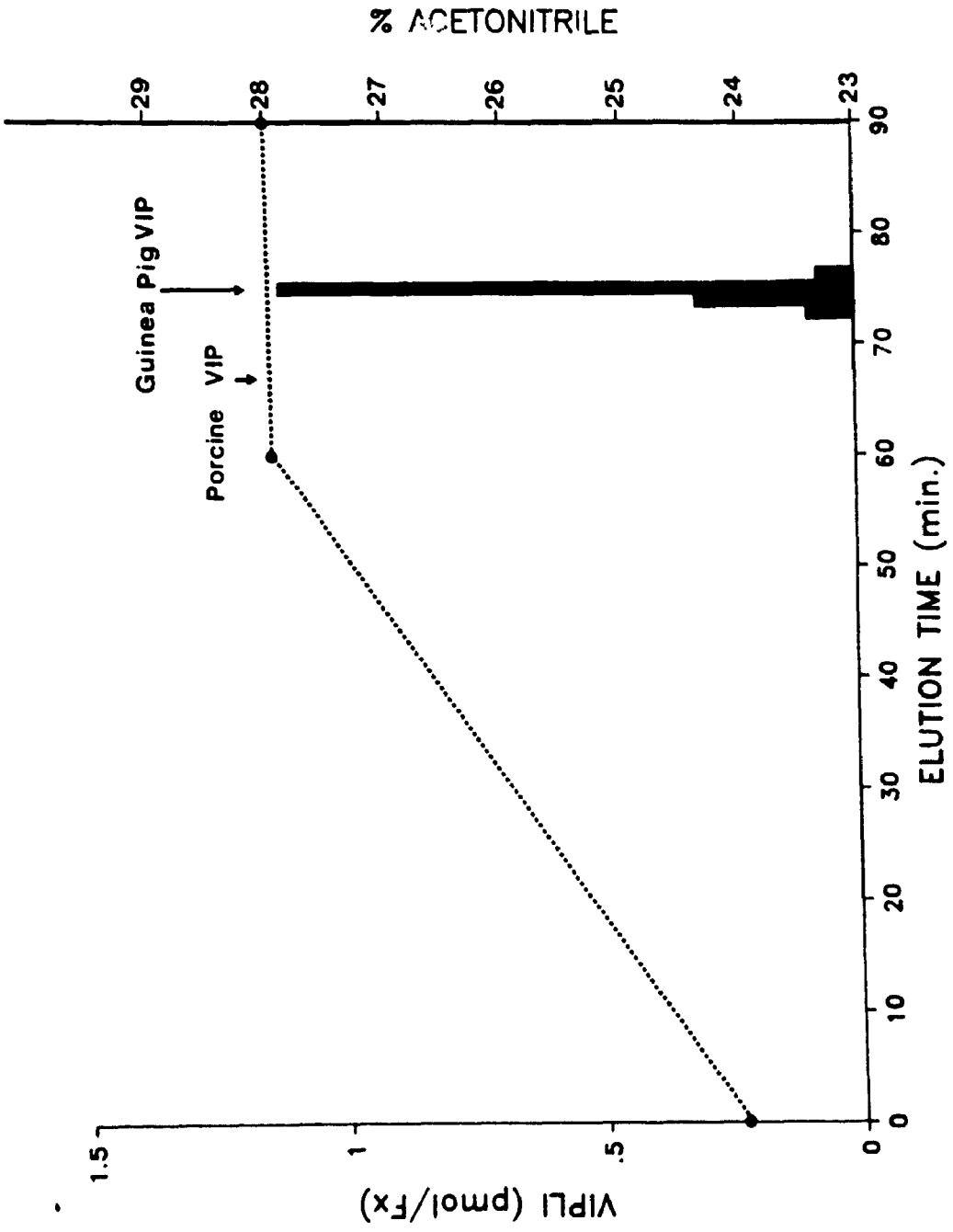

% ACETONITRILE
29
28
27
26
25
24
23
Guinea Pig VIP
Porcine VIP
90
80
70
60
50
40
30
20
10
0
ELUTION TIME (min.)
1.5
1
.5
0
VIPLI (pmol/Fx)

Fig. 36. Cross-reactivity of the a-neurokinin antiserum with several different peptides. Standard curves for the binding of ^{125}I-a-neurokinin to the a-neurokin antiserum are shown with standards a-neurokinin, b-neurokinin, SP, GRP, PYY, Leu-Enk, VIP and Met-Enk. A 3 day incubation period was allowed and a charcoal/dextran separation method was used. This experiment was repeated once with essentially the same results.

Figure 36

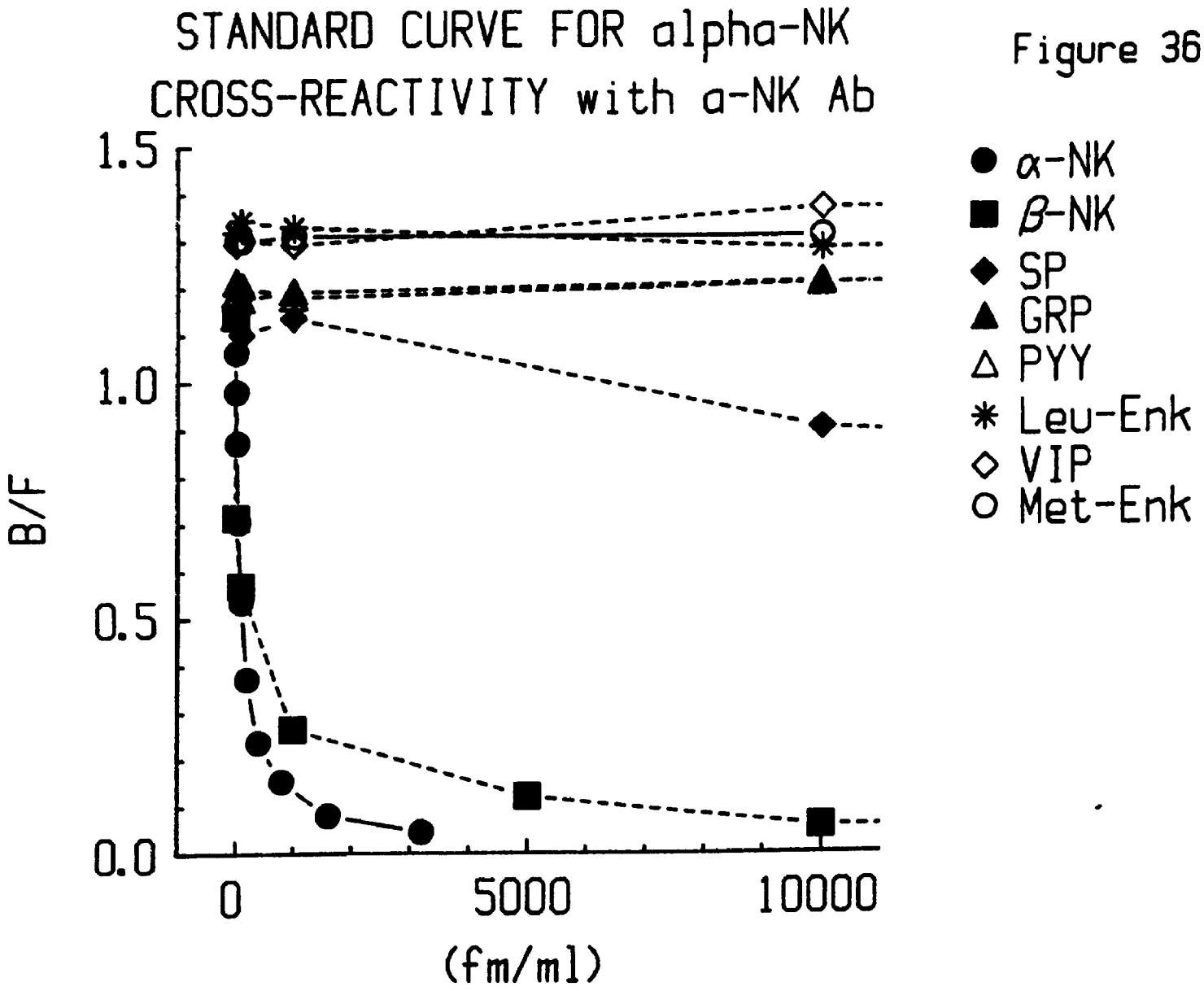

STANDARD CURVE FOR alpha-NK
CROSS-REACTIVITY with a-NK Ab
B/F
1.5
1.0
0.5
0.0
0
5000
10000
(fm/ml)
α-NK
β-NK
SP
GRP
PYY
Leu-Enk
VIP
Met-Enk

Fig. 37. Reverse-phase HPLC characteristics of a) SPLI and b) a-NKLI in P_2 preparations under 'steep' gradient elution conditions (see text for details). The elution positions of synthetic porcine SP and a-NK are indicated by the arrows. 1.0 ml of the P_2 lysate, containing about 750 ug tissue, was injected on the column.

Figure 37

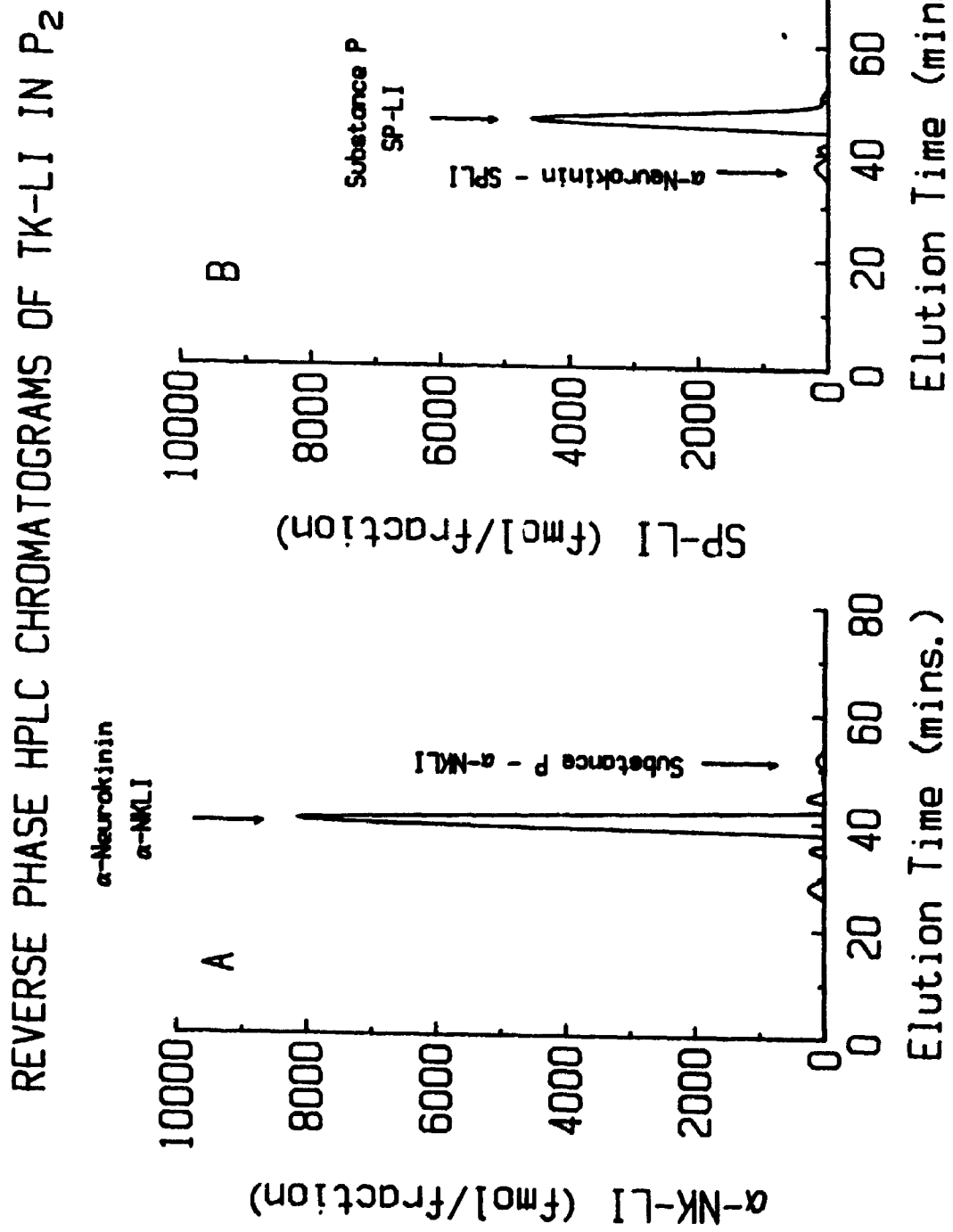

REVERSE PHASE HPLC CHROMATOGRAMS OF TK-LI IN P2
A
α-Neurokinin
α-NKLI
Substance P - α-NKLI
α-NK-LI (fmol/fraction)
10000
8000
6000
4000
2000
0
0
20
40
60
80
Elution Time (mins.)
B
Substance P
SP-LI
α-Neurokinin - SPLI
SP-LI (fmol/fraction)
10000
8000
6000
4000
2000
0
0
20
40
60
80
Elution Time (mins.)

Fig. 38. RIA sample blanks for a) a-NKLI, b) MELI and c) SPLI over a range of dilutions of the P_2 suspension. The zeros for these experiments were 1.1 (a-NKLI), 1.7 (MELI) and 1.6 (SPLI) (See methods for further details).

Figure 38

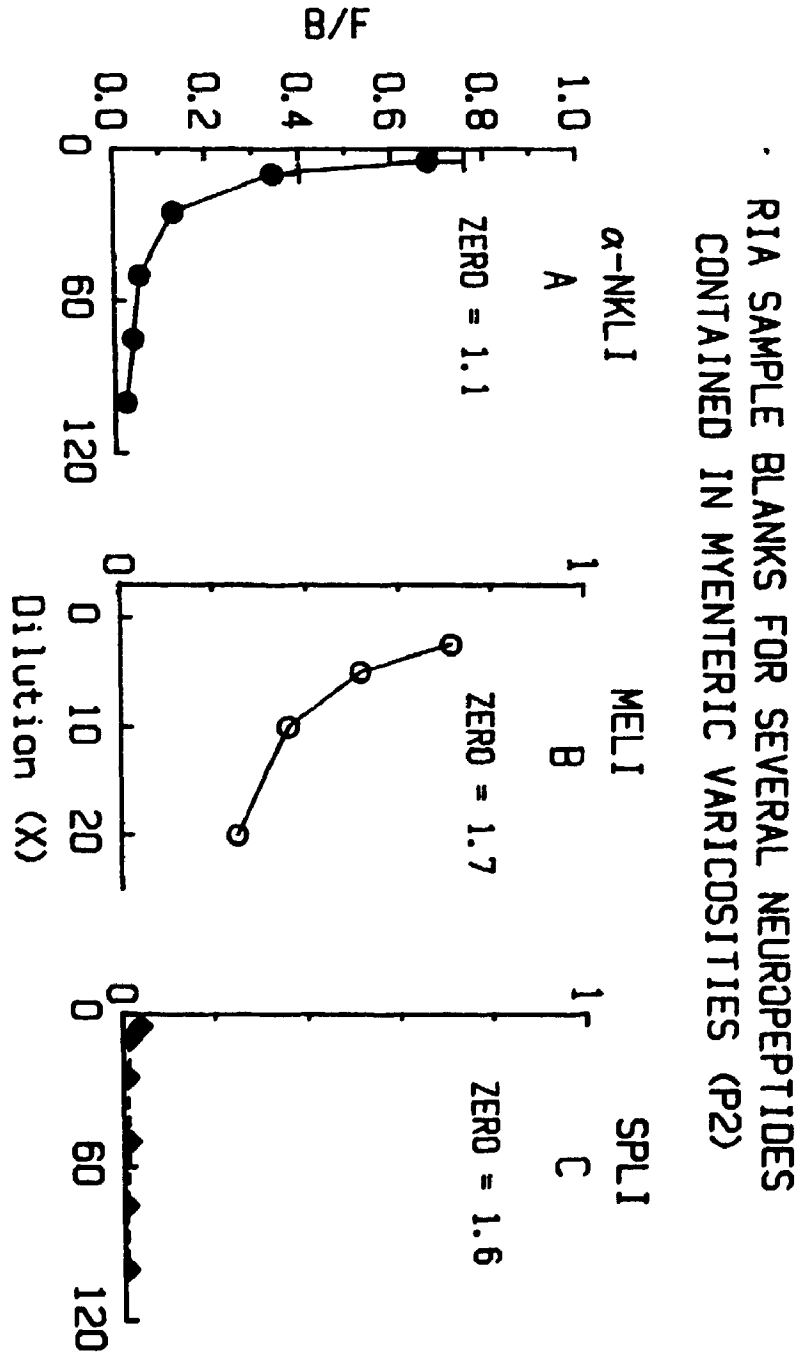

RIA SAMPLE BLANKS FOR SEVERAL NEUROPEPTIDES
CONTAINED IN MYENTERIC VARICOSITIES (P2)
B/F
1.0
0.8
0.6
0.4
0.2
0.0
0
60
120
α-NKLI
A
ZERO = 1.1
1
0
0
10
20
Dilution (X)
MELI
B
ZERO = 1.7
1
0
0
60
120
SPLI
C
ZERO = 1.6

Fig. 39. Degradation of exogenous substance P by P_2 enzymes in the presence or absence of enzyme inhibitors, incubated at 37 C over 60 min. Approximately 50-75 ug of tissue was contained in each incubation mixture.

a) Shows the control degradation curve for 36nM exogenous substance P. Bars represent the S.E.M values from triplicate determinations from one of three representative experiments. The inset shows the pseudo-first-order rate plot of the degradation reaction. The line through the data points is the least squares regression line (r_2 =-.997) with slope -.077 $\pm$ .0037.

b) Shows the pseudo-ist order rate plots, for control degradation, in the presence of .5mM bacitracin or in the presence of .5mM bacitracin, .25mM leupeptin and 25 ug/ml chymostatin. The least squares regression lines through the points had slopes of .0269 $\pm$ .00019 (r=.991) in the presence of bacitracin alone and -.02374 $\pm$ .0014 (r=-.993) in the presence of the mixture. The rates of degradation are: 1600 pmoles/min for the control (slope = -.02686) 402 pmoles/min in the presence of bacitracin alone and 928 pmoles/min in the presence of bacitracin, leupeptin and chymostatin. Rates all significantly different from each other ($p < .05$).
The experiments in a) and b) were repeated twice more with similar results.

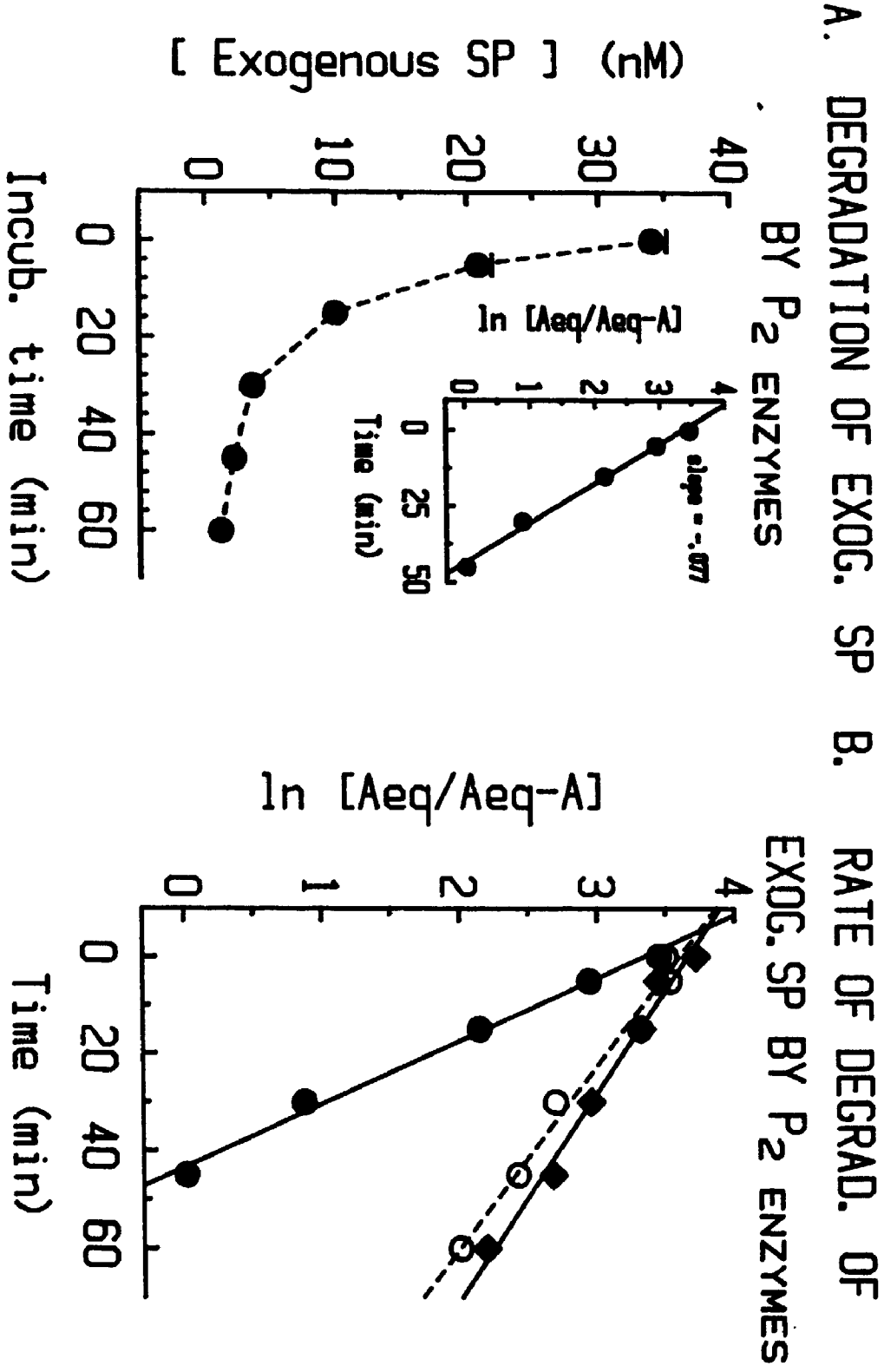

A. DEGRADATION OF EXOG. SP BY P2 ENZYMES
[Exogenous SP] (nM)
0
10
20
30
40
Incub. time (min)
0 20 40 60
ln [Aeq/Aeq-A]
0 1 2 3 4
Time (min)
0 25 50
B. RATE OF DEGRAD. OF EXOG. SP BY P2 ENZYMES
ln [Aeq/Aeq-A]
0 1 2 3 4
Time (min)
0 20 40 60

Figure 39

Fig. 40. Effect of equilbration time on the neuronal stores of SPLI in PV preparations suspended in Krebs buffer (pH 7.4; gassed with 95% O_2/5% CO_2) at 37 C. The line drawn through the points is the least squares regression line (r=.997). The bars represent the S.E.M. values from 4 separate experiments.

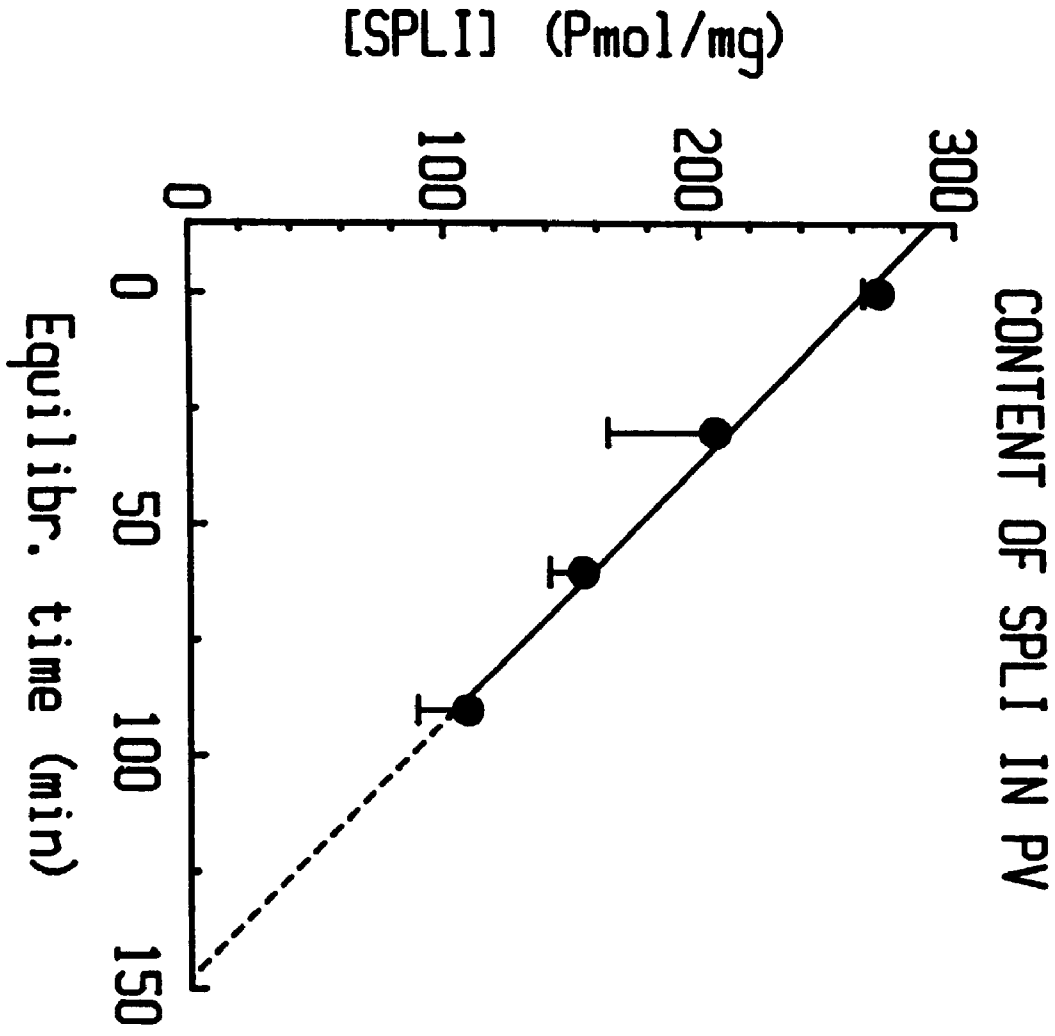

[SPLI] (Pmol/mg)
0
100
200
300
Equilibr. time (min)
0
50
100
150
CONTENT OF SPLI IN PV

Figure 40

Fig. 41. a) Basal release of SPLI throughout the equilibration of the P_2 suspensions in Hepes buffered medium (pH 7.2) at 37 C. The bars represent the mean $\pm$ S.E.M. values from 4 separate experiments. Time zero represents the time at which the P_2 pellets were resuspended in buffer and placed at 37 C for equilibration.

b) Effect of the suspension buffer on the basal release of SPLI from P_2. The P_2 was: isolated in 0.32M sucrose and resuspended in Hepes buffered medium (S/H), isolated and resuspended in Hepes buffered medium (H/H), isolated in 0.32M sucrose and resuspended in gassed Krebs (S/K) or isolated and resuspended in gassed Krebs (K/K). The bars represent the mean $\pm$ S.E.M. from 4 preparations isolated and resuspended separately at the same time during the same experiment.

Basal release was determined following a 60 min equilbration time at 37 C.

The neuronal content of SPLI in these preparations was 20 pmoles/mg (S/H), 5.5 pmoles/mg (H/H), 22 pmoles/mg (S/K) and 7 pmoles/mg (S.E.M < 4%). Significant differences exist in the neuronal contents between those preparation isolated in Hepes/Krebs buffer and those isolated in 0.32M sucrose ($p < .05$).

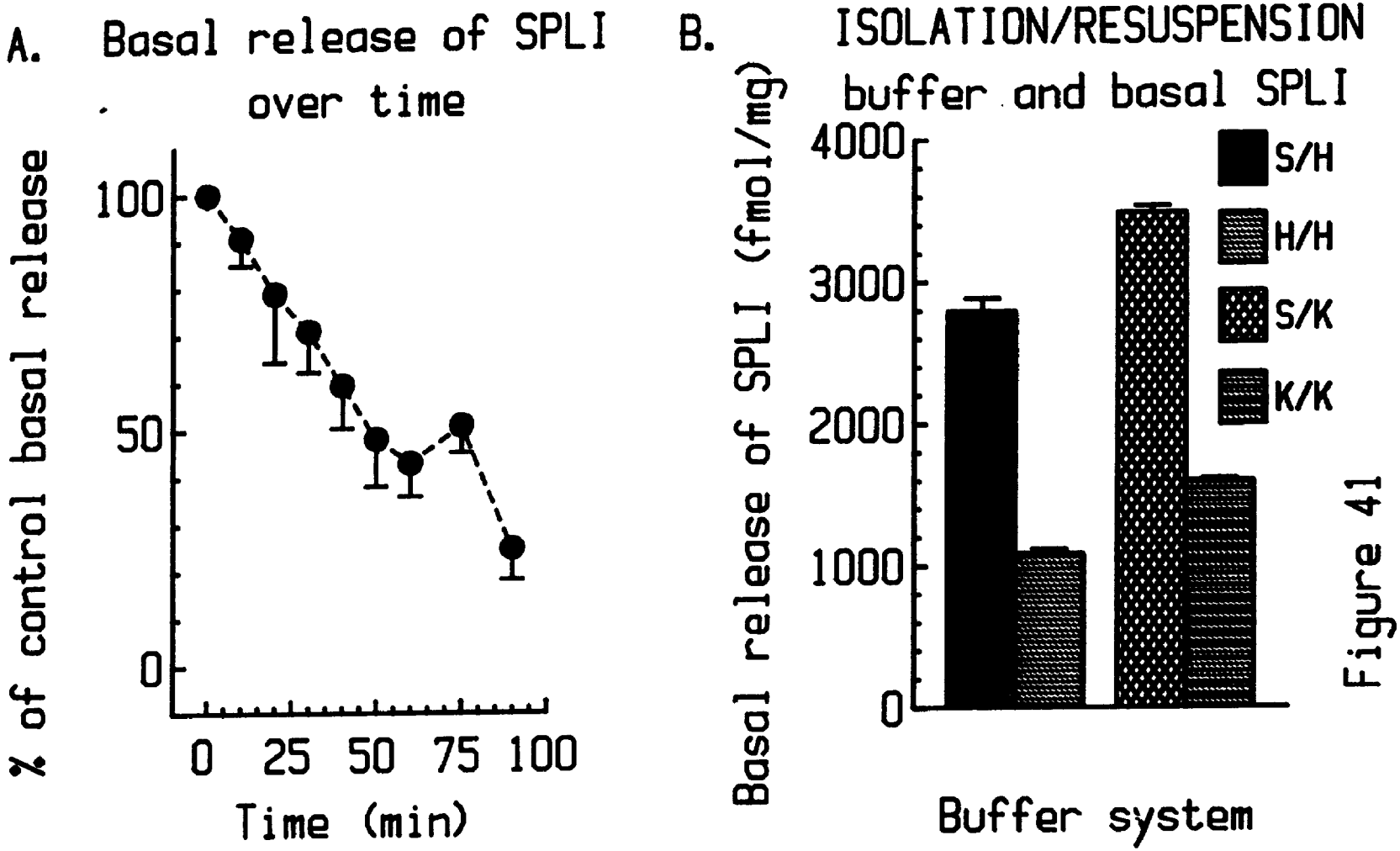

A.
Basal release of SPLI over time
% of control basal release
100
50
0
0 25 50 75 100
Time (min)
B.
ISOLATION/RESUSPENSION buffer and basal SPLI
Basal release of SPLI (fmol/mg)
4000
3000
2000
1000
0
S/H
H/H
S/K
K/K
Buffer system

Figure 41

Fig. 42. a) Effect of bacitracin on the basal release of SPLI from isolated myenteric varicosities (P_2). Following a 60 min equilibration of the P_2 suspensions at 37 C, in either Krebs (gassed,pH 7.4) or Hepes buffered medium (pH 7.2) containing incrementing concentrations of bacitracin (pre-incubated for 7 min with P_2),the suspensions were centrifuged (13,000 X g) for 1min, boiled and then the supernatants were cooled to room temp. and frozen until assayed. The bars represent the mean $\pm$ the S.E.M. from 4 P_2 preparations. The curves were drawn as the least squares regression line (Krebs resuspension; r=.9934) or by joining the points (Hepes resuspension). b) The effect of bacitracin on the basal release of SPLI from P_2. The bar represents the mean $\pm$ S.E.M. from 4 preparations.

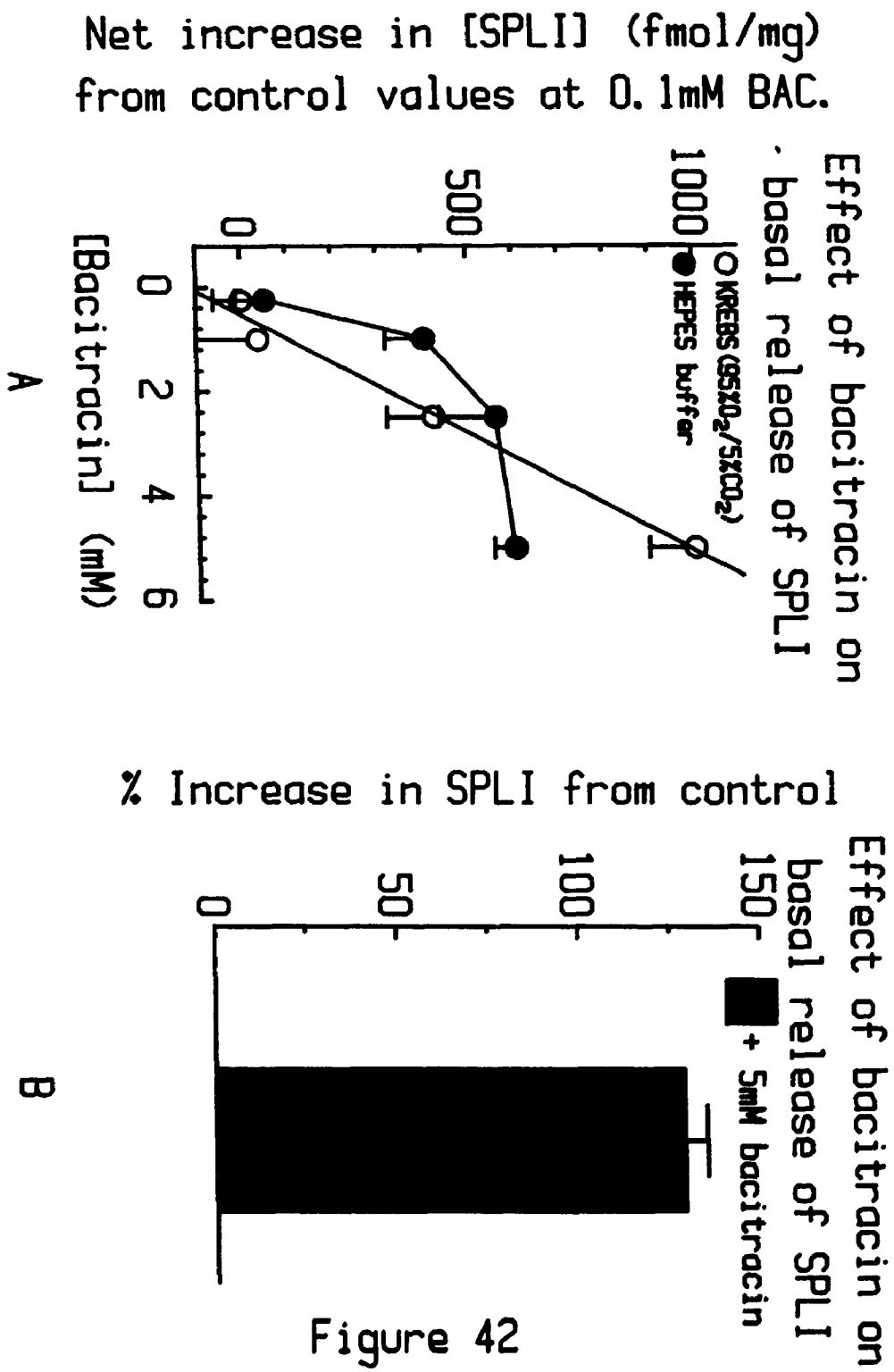

Net increase in [SPLI] (fmol/mg)
from control values at 0.1mM BAC.
Effect of bacitracin on
basal release of SPLI
1000
500
0
KREBS (95%O2/5%CO2)
HEPES buffer
0 2 4 6
[Bacitracin] (mM)
A
% Increase in SPLI from control
Effect of bacitracin on
basal release of SPLI
150
100
50
0
+ 5mM bacitracin
B

Figure 42

Fig. 43. The Concentration of cations in the S_3 and S_4 supernatants obtained during the isolation of the P_2 fraction (for details of methodology refer to methods). The concentrations of $[Ca^{++}]$ and $[Mg^{++}]$ in the supernatants were measured by atomic absorption spectrophotometry and that for $[K^{+}]$ was measured by atomic emission. The results represent the mean $\pm$ S.E.M. values from 4 experiments.

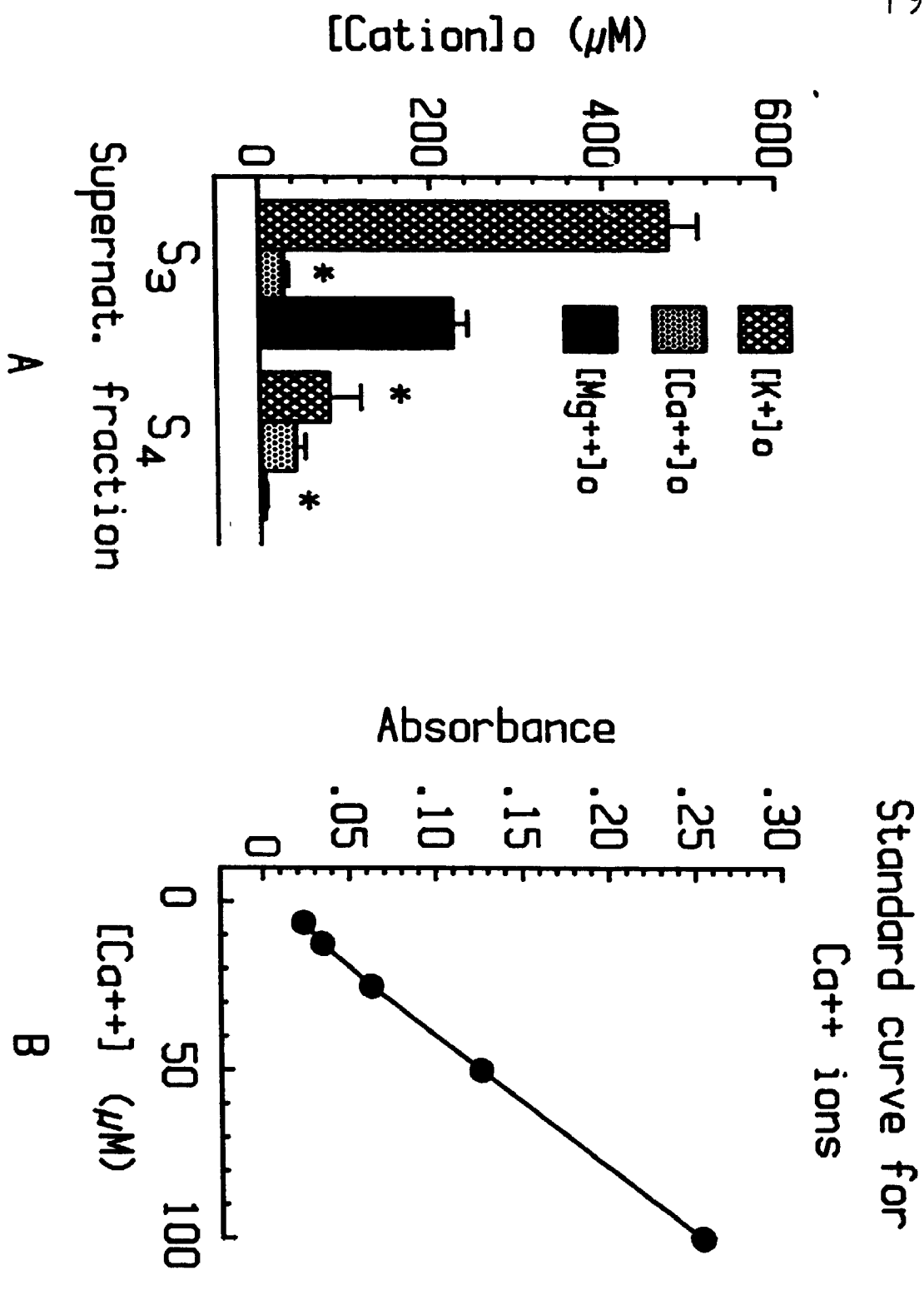

[Cation]o (μM)
0
200
400
600
[K+]o
[Ca++]o
[Mg++]o
S3
S4
Supernat. fraction
A
Absorbance
0
.05
.10
.15
.20
.25
.30
Standard curve for
Ca++ ions
0
50
100
[Ca++] (μM)
B

Figure 43

Fig. 44. Effect of centrifugation speed on the basal release of tachykinins from isolated myenteric varicosities (P_2). The basal release of SPLI and a-NKLI was expressed as a percentage of the basal release observed following the control centrifugation speed (13,000 X g). The S_4 represents the supernatant which was obtained after ultracentrifugation of the S_3 (105,900 X g). The P_u represents the pellet resulting from the ultracentrifugation of the S_3. The results represent the mean $\pm$ S.E.M. from 6 P_2 samples.

* significant differences from values obtained for S_4 for both SPLI and a-NKLI.

Figure 44

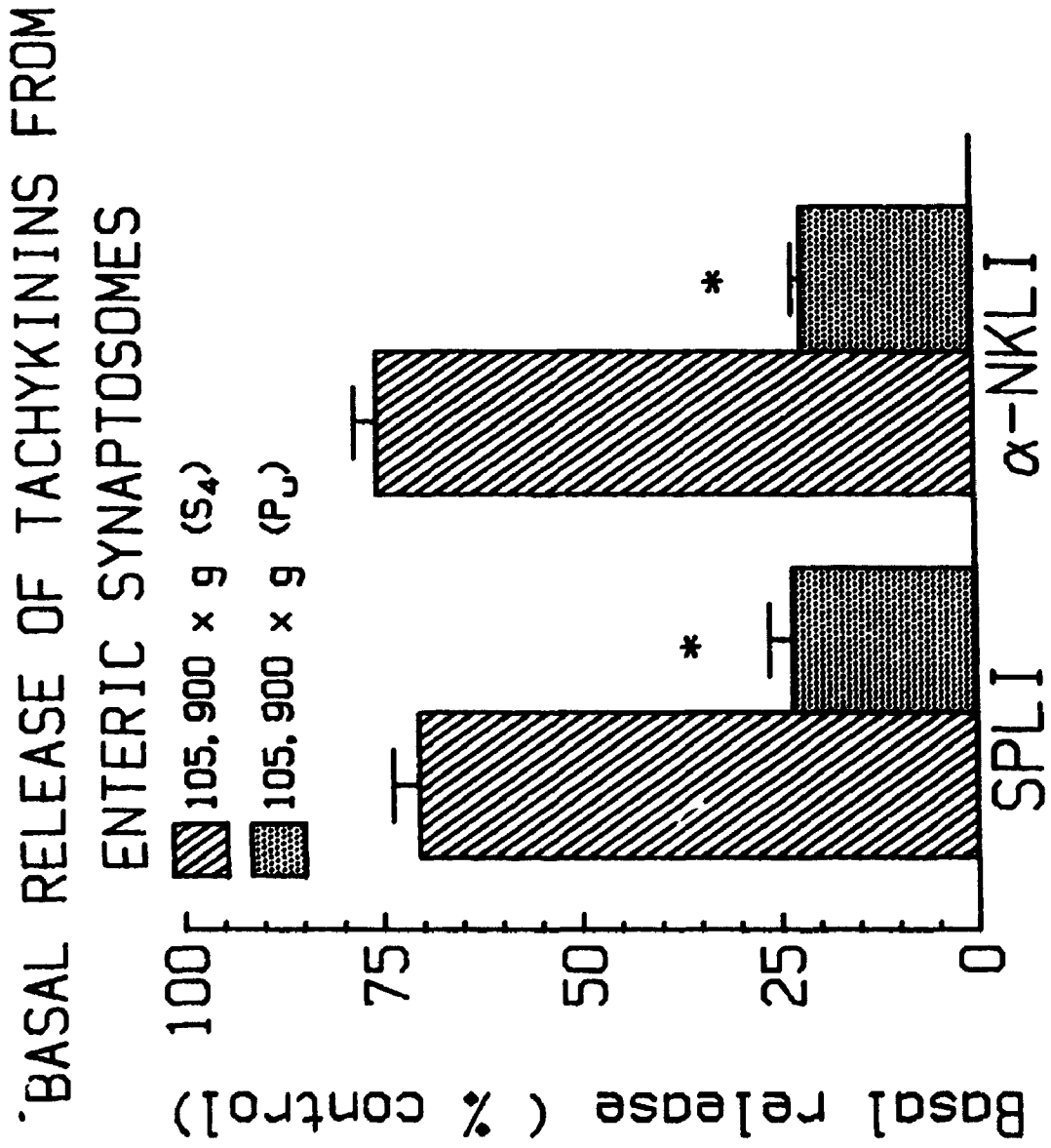

BASAL RELEASE OF TACHYKINS FROM ENTERIC SYNAPTOSOMES
105,900 x g (S4)
105,900 x g (P3)
Basal release (% control)
100
75
50
25
0
*
*
SPLI
α-NKLI

Fig. 45. Effect of centrifugation speed on the values of sample blanks for a) SPLI and b) a-NKLI in several fractions obtained during the isolation of the P_2. Sample blanks were determined for SPLI, in supernatants obtained with both low speed centrifugal separation [ie. at 13,000 X g (S_3)] and high speed centrifugal separation [105,900 X g (S_4)]. Sample blanks were also determined for the pellet obtained with the high speed centrifugal separation (P_u). The sample blanks were measured for several sample dilutions and the values represent the means $\pm$ S.E.M from 6 P_2 samples.

Figure 45a

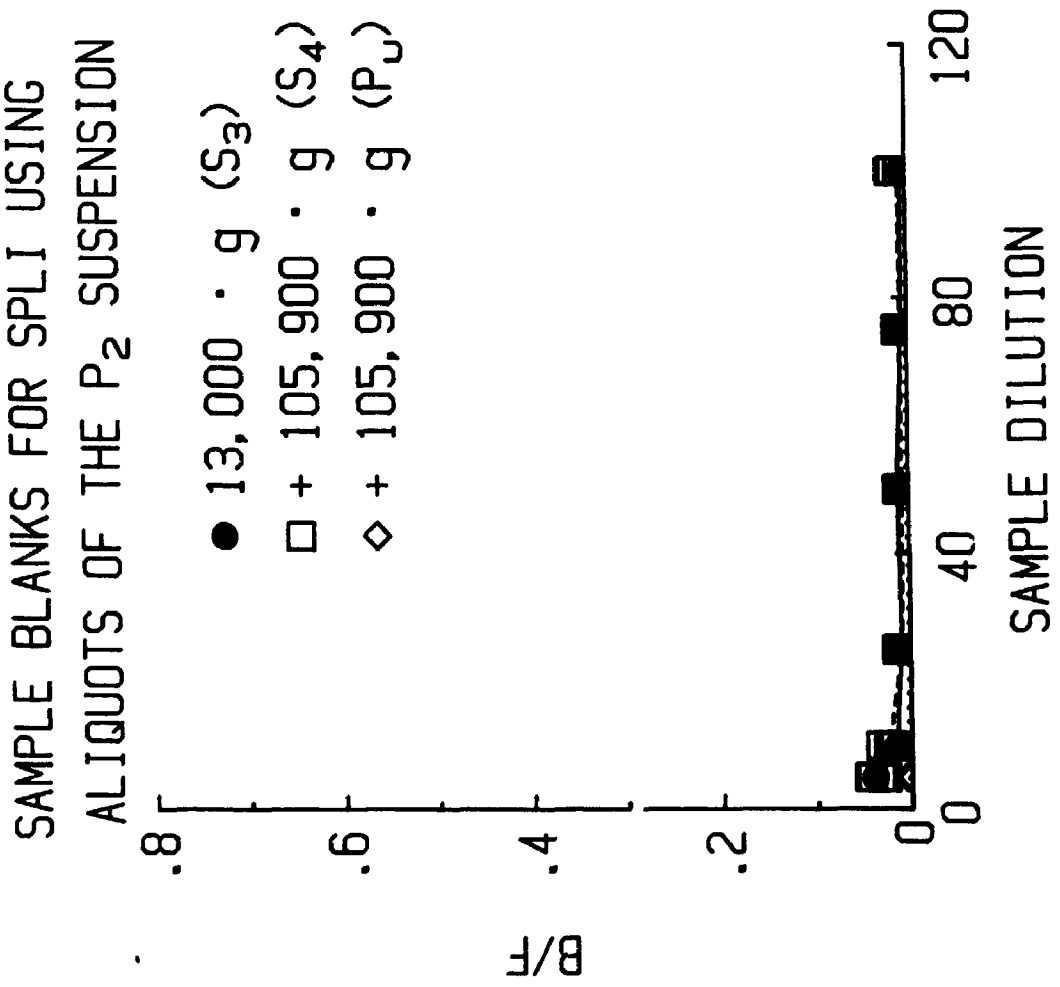

SAMPLE BLANKS FOR SPLI USING
ALIQUOTS OF THE P_2 SUSPENSION
● 13,000 · g (S_3)
□ + 105,900 · g (S_4)
◇ + 105,900 · g (P_3)
B/F
.8
.6
.4
.2
0
0
40
80
120
SAMPLE DILUTION

Figure 45b

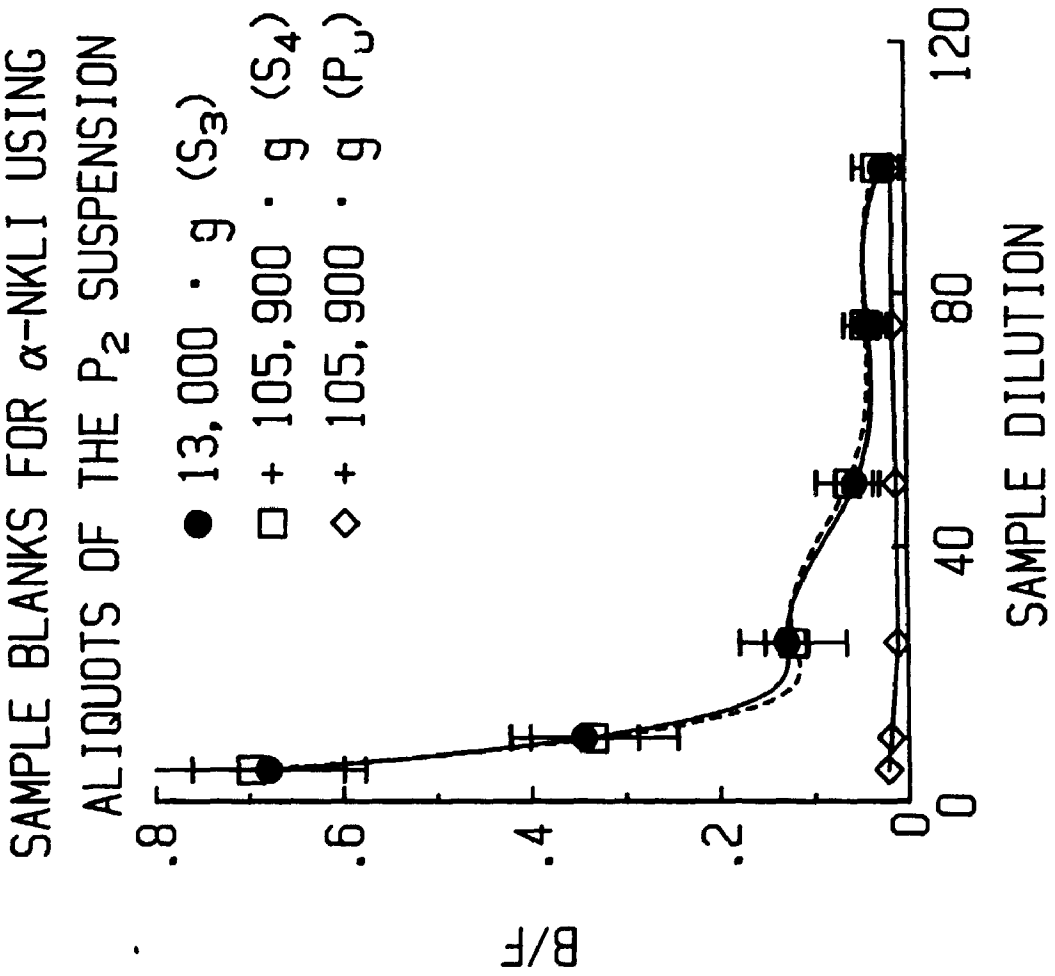

SAMPLE BLANKS FOR α-NKLI USING
ALIQUOTS OF THE P2 SUSPENSION
● 13,000 · g (S3)
□ + 105,900 · g (S4)
◇ + 105,900 · g (PJ)
B/F
.8
.6
.4
.2
0
0
40
80
120
SAMPLE DILUTION

Fig. 46. Dose-dependent release of TKLI's (ie. SPLI and a-NKLI) from isolated myenteric varicosities (P_2) with the Na^+ ionophore monensin. The synaptosomes were resuspended in Hepes buffered medium (pH 7.2), equilibrated at 37 C for 60 min. and then preincubated with bacitracin (5mM) for 7 min. The 3 min. release reaction was initiated by adding 700 ul of P_2 suspension to incubation tubes containing various concentrations of monensin. These are the results from a single experiment. Essentially identical results were obtained in one other experiment. Approximately 0.75 mg tissue was used per incubation tube.

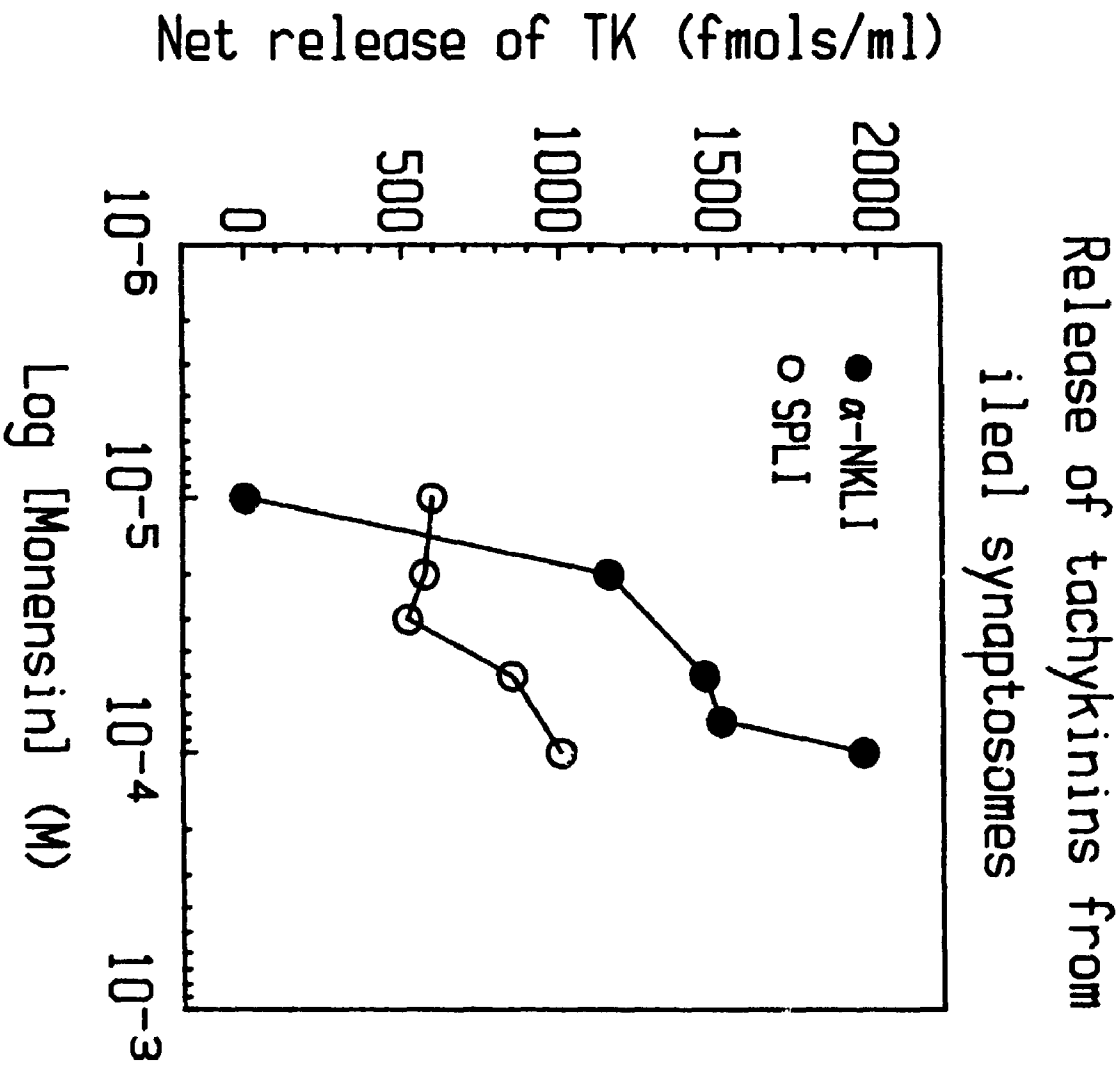

Release of tachykinins from ileal synaptosomes
Net release of TK (fmols/ml)
0
500
1000
1500
2000
Log [Monensin] (M)
10-6
10-5
10-4
10-3
● α-NKLI
○ SPLI

Figure 46

Fig. 47. Release of SPLI and GALLI from isolated myenteric varicosities (P_2) with the depolarizing stimulus K^+. The synaptosomes were resuspended in Hepes buffered medium (pH 7.2), equilibrated at 37 C for 60 min. and then preincubated with bacitracin (5mM) for 7 min. The 3 min. release reaction with K^+ was initiated by adding 700 ul of P_2 suspension to incubation tubes containing various concentrations of K^+. The release with K^+ was determined in the presence or absence of several different antagonists; Release of GALLI was not detectable under any of the conditions [N.B.: no release of SPLI was observed in the absence of any antagonists (n=6)]. The antagonists were preincubated with the tissue (suspended in Locke's solution) for 45-60 min. The results represent the mean responses from 3 separate experiments. The standard errors for individual data points are < 5%. Approximately 0.75mg tissue was used per incubation tube.

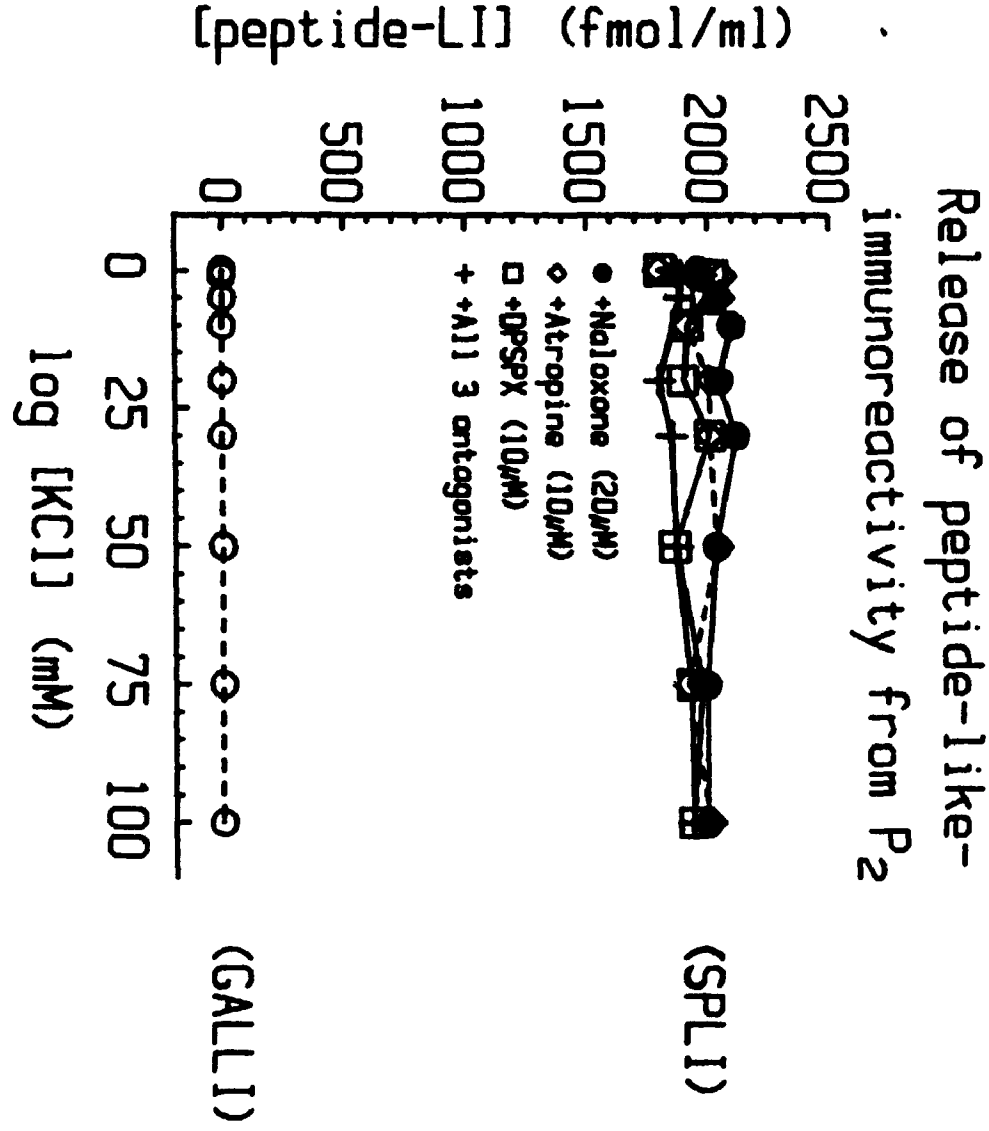

Release of peptide-like-immunoreactivity from P2
[peptide-LI] (fmol/ml)
2500
2000
1500
1000
500
0
● +Naloxone (20μM)
◇ +Atropine (10μM)
□ +DPSPX (10μM)
+ +All 3 antagonists
(SPLI)
0 25 50 75 100
log [KCl] (mM)
(GALLI)

Figure 47

Fig. 48. Release of a-neurokinin from isolated myenteric varicosities (P_2) with the depolarizing stimulus K^+. The synaptosomes were resuspended in Locke's solution (pH 7.2), equilibrated at 37 C for 60 min. and then preincubated with bacitracin (5mM) for 7 min. The 3 min. release reaction with K^+ was initiated by adding 700 ul of P_2 suspension to incubation tubes containing various concentrations of K^+. The release with K^+ was determined in the presence or absence of several different antagonists [N.B.: no release of a-NK was observed in the absence of any antagonists (n=6)]. The antagonists were pre-incubated with the tissue for 45-60 min. The results represent the mean responses from 3 separate experiments. The standard errors for individual data points are < 5%. Approximately 0.75mg tissue was used per incubation tube.

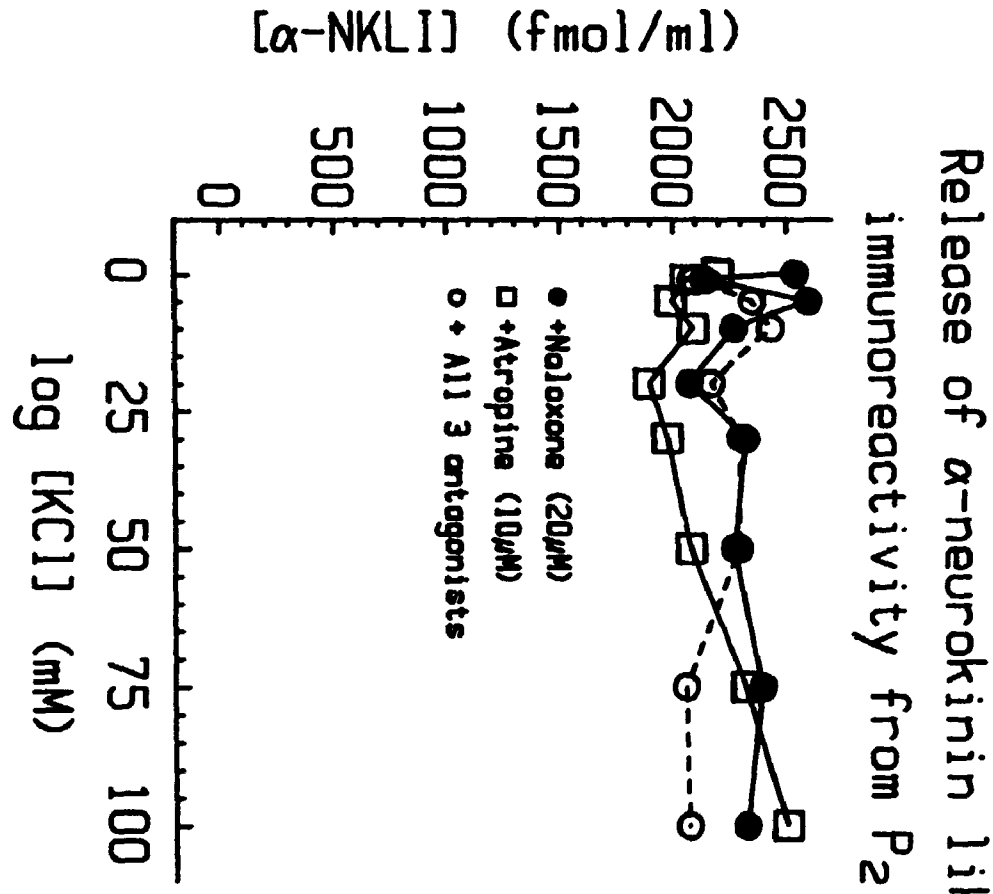

Release of α-neurokinin like immunoreactivity from P2
[α-NKLI] (fmol/ml)
2500
2000
1500
1000
500
0
0 25 50 75 100
log [KCl] (mM)
● +Naloxone (20μM)
□ +Atropine (10μM)
○ + All 3 antagonists

Figure 48

Fig. 49. Release of a-neurokinin-LI from isolated myenteric varicosities (P_2) with the depolarizing stimulus K^+. The release was measured in the presence of the A_1-selective adenosine receptor antagonists (DPSPX). Each dose-response curve represents the findings from a single experiment. The intra-assay variability is < 5%). (Details of the release protocal as in fig. 48). Approximately, 0.75 mg tissue was used per incubation tube.

Figure 49

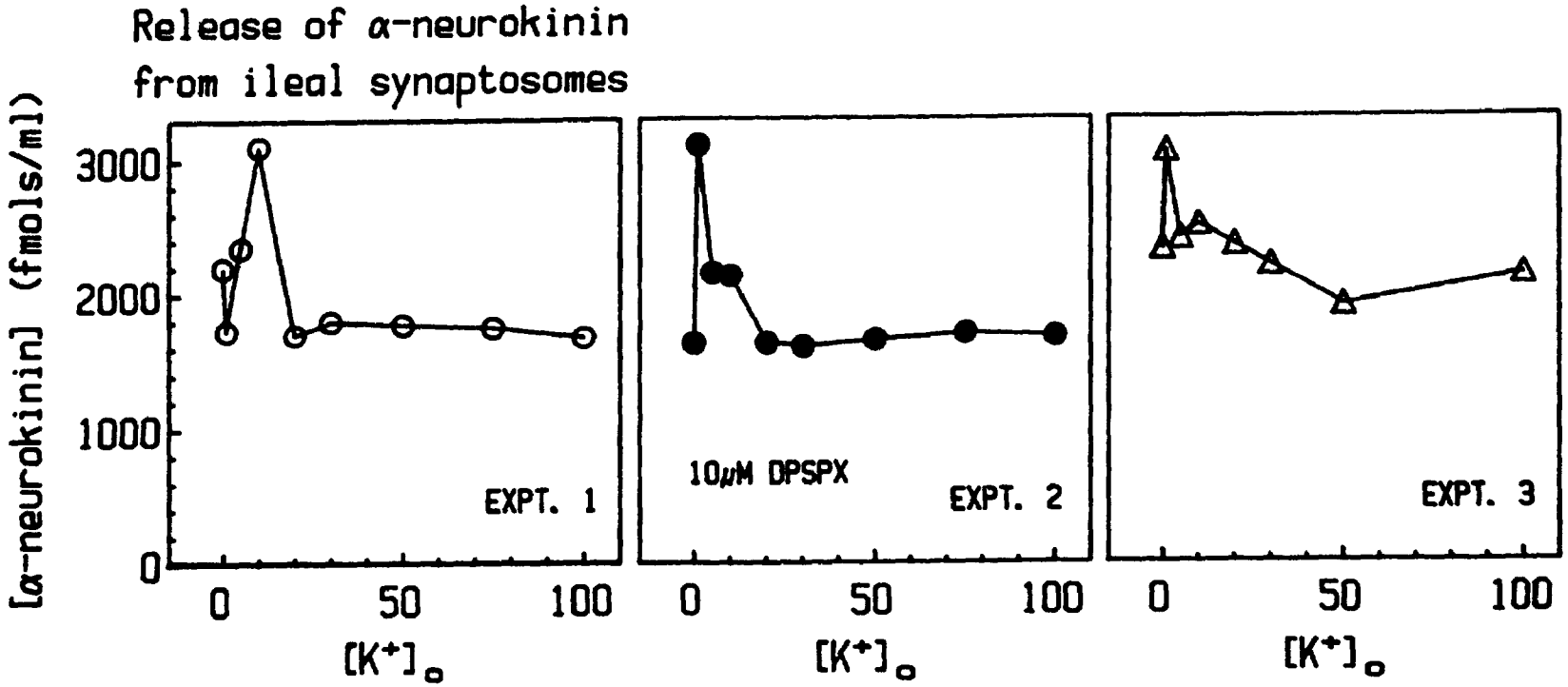

Release of α-neurokinin
from ileal synaptosomes
[α-neurokinin] (fmols/ml)
3000
2000
1000
0
EXPT. 1
10μM DPSPX
EXPT. 2
EXPT. 3
0
50
100
[K+]o

Fig. 50. Release of MELI from isolated myenteric varicosities (P_2) by the depolarizing stimulus K^+ a) in the presence of the opioid antagonist naloxone, b) atropine, c) DPSPX or d) naloxone, atropine and DPSPX. Dose-response curves are shown for 3 separate experiments at 3 different dilutions of the samples which were used in the RIA. Approximately 0.75 mg tissue was used per incubation tube. A 3 min release reaction was used following first a 45-60 min equilibration with naloxone in Locke's solution and then a 7 min equilibration with bacitracin (5mM) at 37 C. The intra-assay variability at each dilution is < 5%.

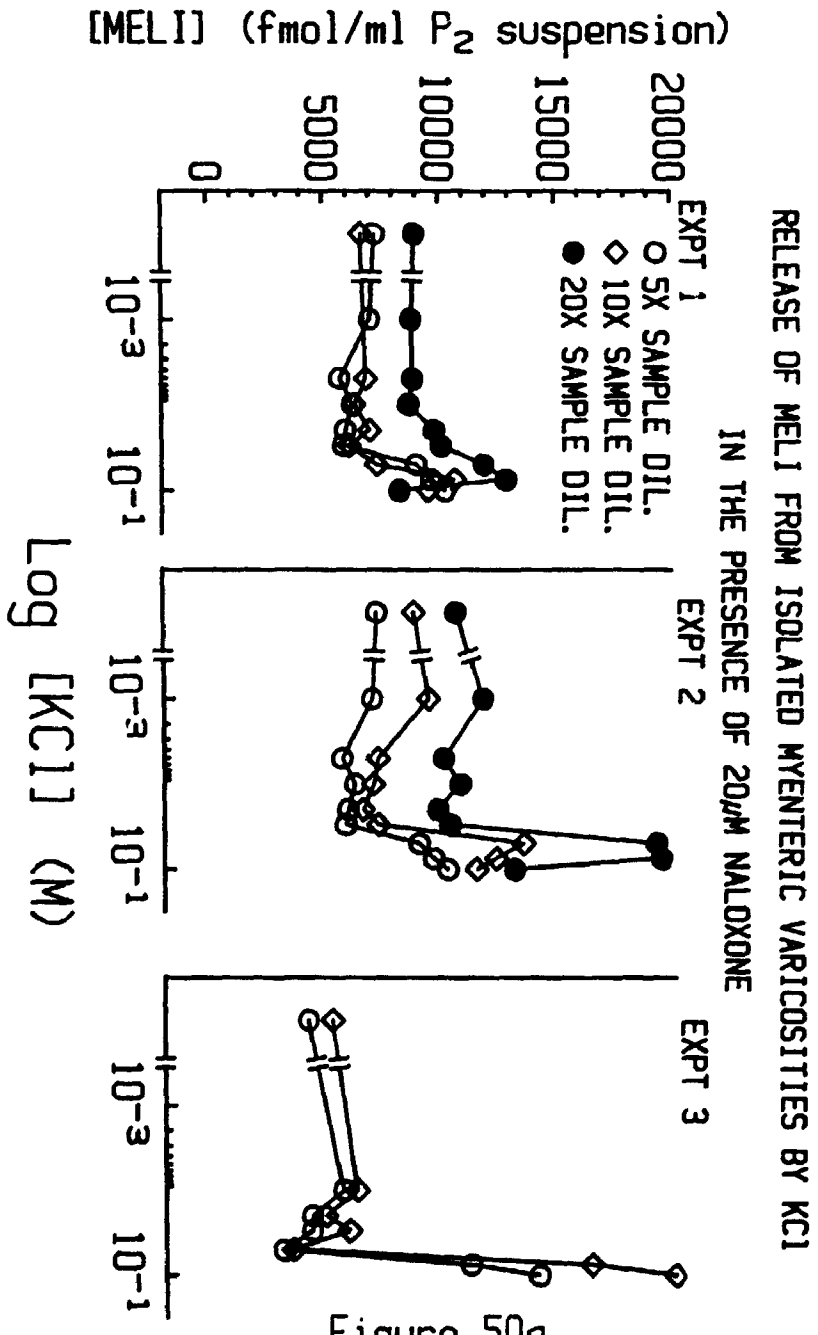

RELEASE OF MELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 20μM NALOXONE
[MELI] (fmol/ml P2 suspension)
20000
15000
10000
5000
0
EXPT 1
○ 5X SAMPLE DIL.
◇ 10X SAMPLE DIL.
● 20X SAMPLE DIL.
EXPT 2
EXPT 3
10-3
10-1
Log [KCl] (M)

Figure 50a

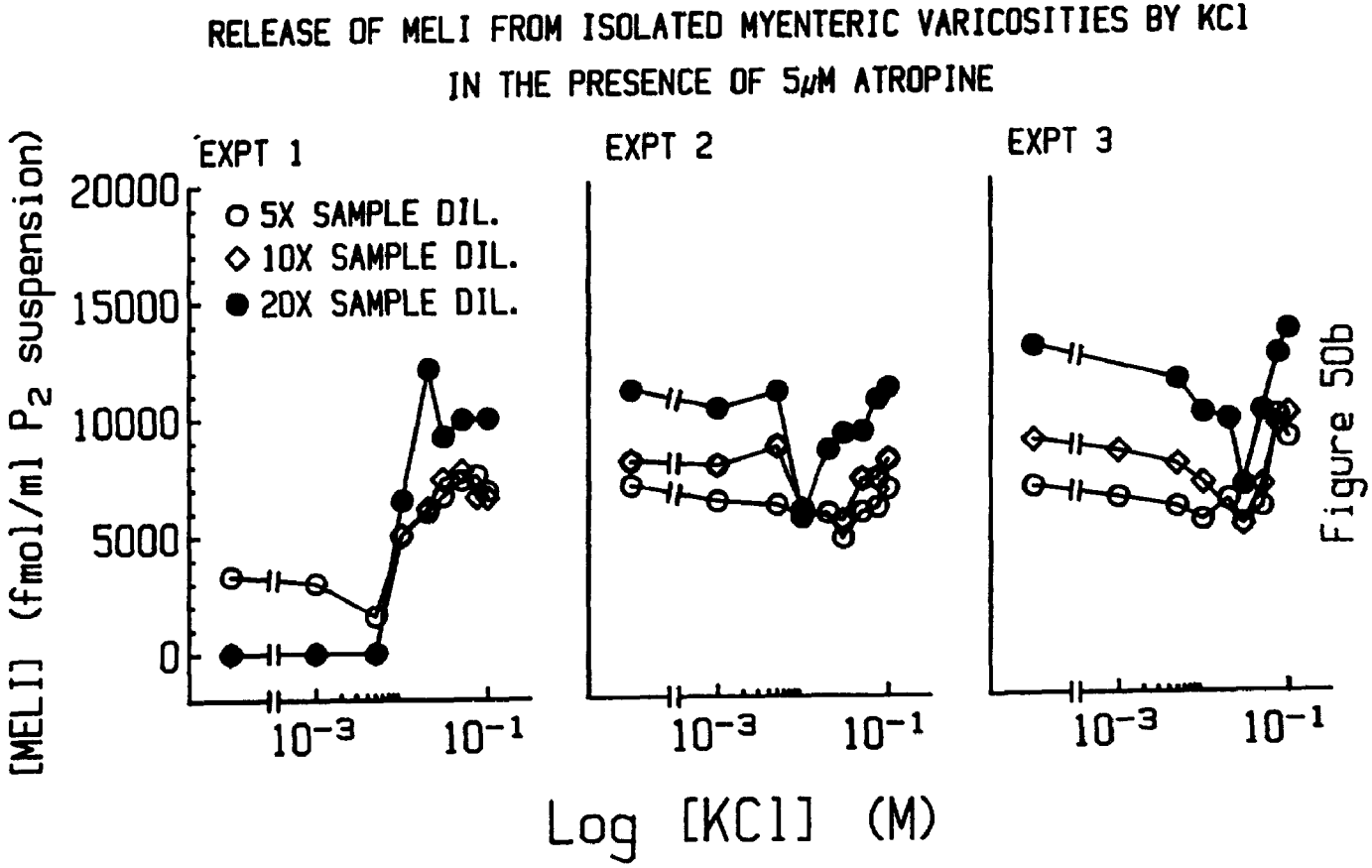

RELEASE OF MELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 5μM ATROPINE
EXPT 1
EXPT 2
EXPT 3
○ 5X SAMPLE DIL.
◇ 10X SAMPLE DIL.
● 20X SAMPLE DIL.
[MELI] (fmol/ml P2 suspension)
20000
15000
10000
5000
0
10-3
10-1
Log [KCl] (M)

Figure 50b

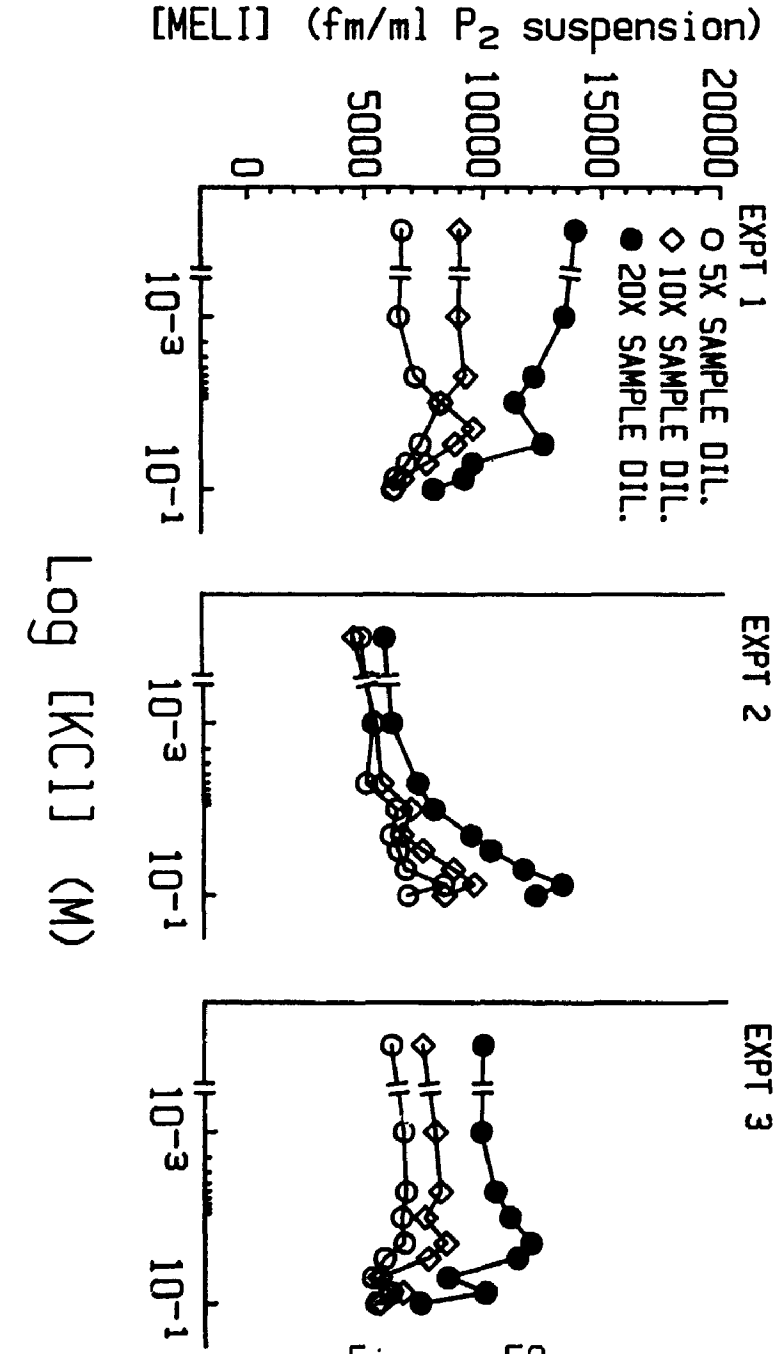

RELEASE OF MELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 10μM 1,3-dipropyl-8-[4-sulfophenyl]-xanthine
[MELI] (fm/ml P2 suspension)
20000
15000
10000
5000
0
EXPT 1
O 5X SAMPLE DIL.
◇ 10X SAMPLE DIL.
● 20X SAMPLE DIL.
EXPT 2
EXPT 3
10-3
10-1
Log [KCl] (M)

Figure 50c

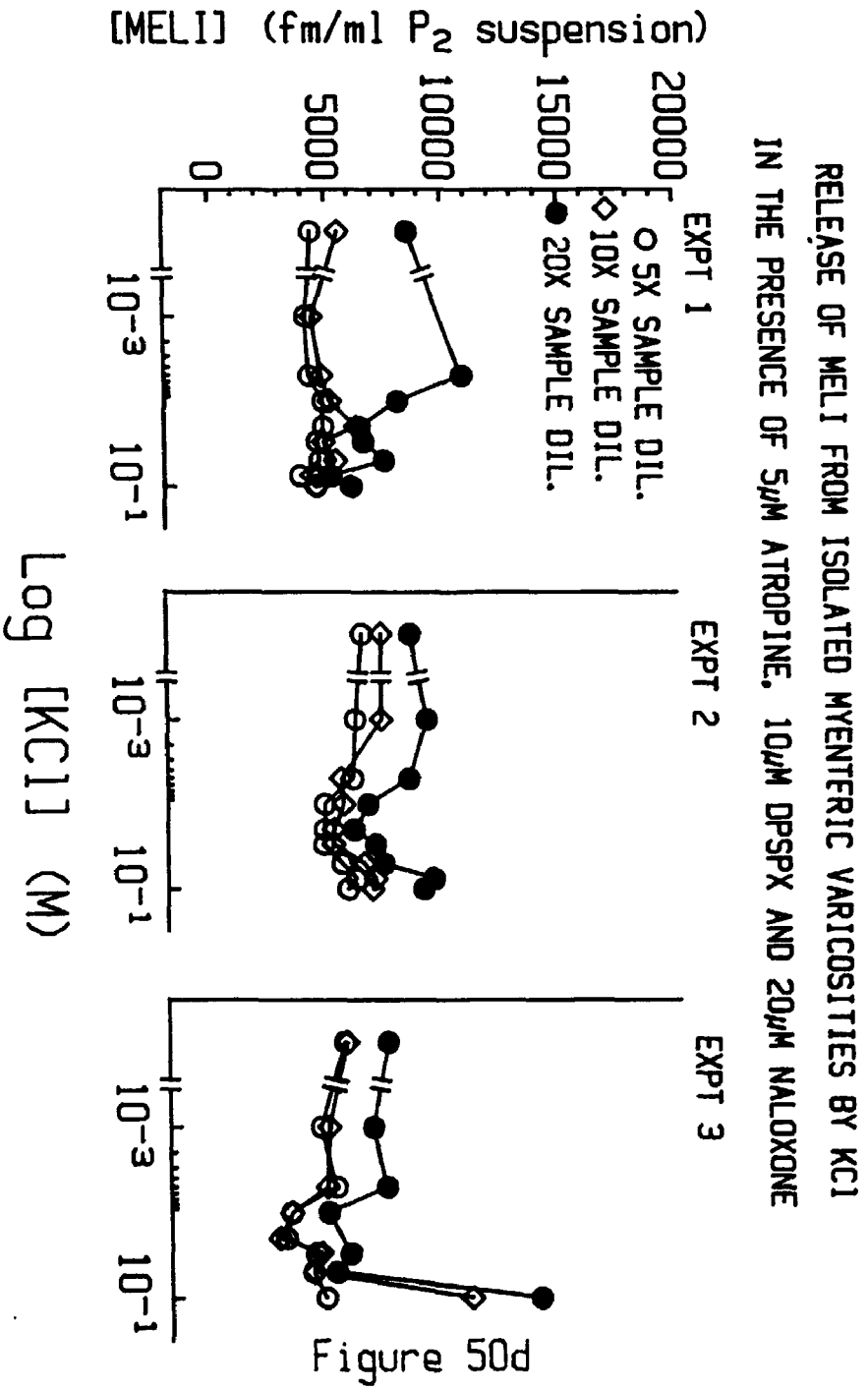

RELEASE OF MELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 5μM ATROPINE. 10μM DPSPX AND 20μM NALOXONE
[MELI] (fm/ml P2 suspension)
20000
15000
10000
5000
0
EXPT 1
O 5X SAMPLE DIL.
◇ 10X SAMPLE DIL.
● 20X SAMPLE DIL.
EXPT 2
EXPT 3
10-3
10-1
Log [KCl] (M)

Figure 50d

Fig. 51. Release of LELI from isolaced myenteric varicosities (P_2) by the depolarizing stimulus K^+ in the presence of a) atropine, naloxone and DPSPX, b) atropine, c) naloxone or d) DPSPX. Dose-response curves are shown for 3 separate experiments at 2 different dilutions of the samples (ie. 10 and 20 X) which were used in the RIA. Approximately 0.75 mg tissue was used per incubation tube. A 3 min release reaction was used following first a 45-60 min equilibration with naloxone in Locke's solution and then a 7 min equilibration with bacitracin (5mM) at 37 C. The intra-assay variability at each dilution is < 5%. Approximately, .75 mg of tissue was used in each incubation tube.

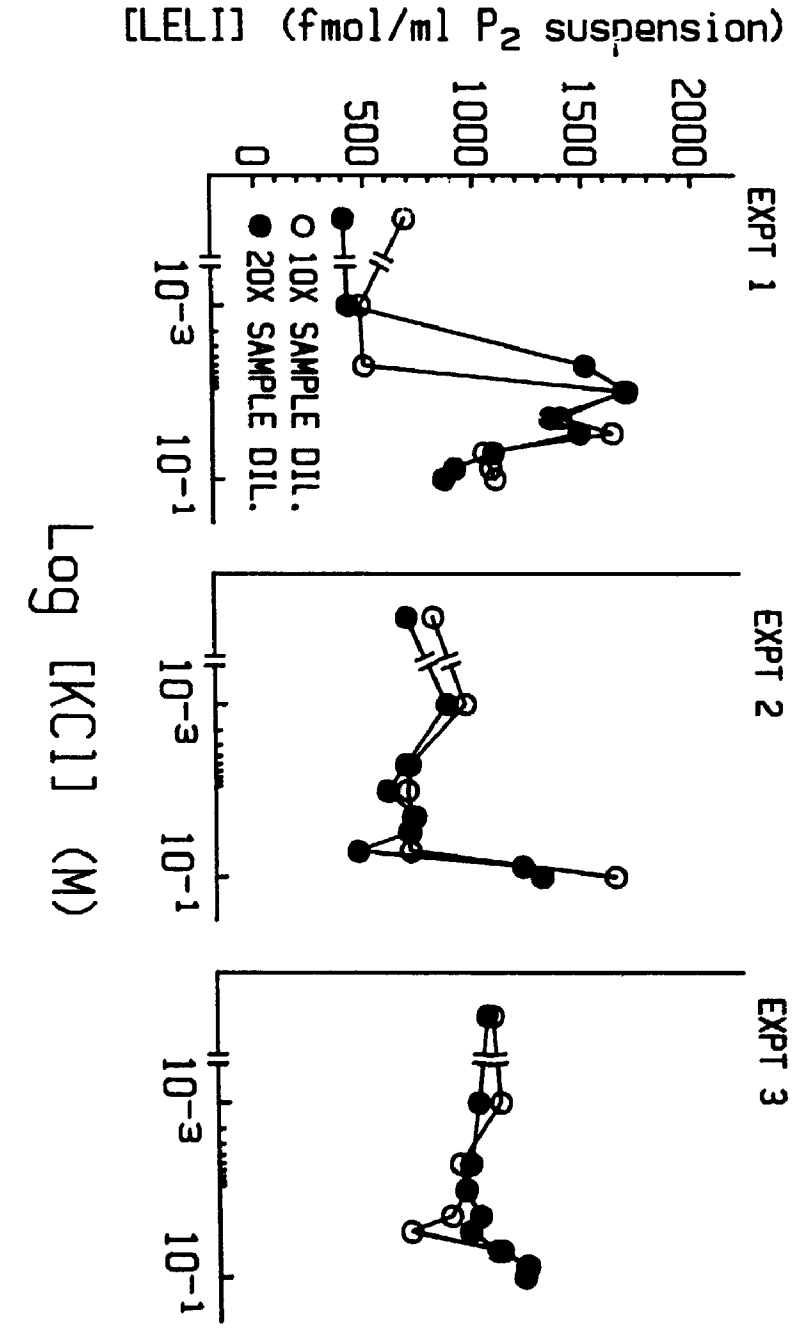

RELEASE OF LELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 5μM ATROPINE, 10μM DPSPX AND 20μM NALOXONE
[LELI] (fmol/ml P2 suspension)
2000
1500
1000
500
0
EXPT 1
EXPT 2
EXPT 3
○ 10X SAMPLE DIL.
● 20X SAMPLE DIL.
10-3
10-1
Log [KCl] (M)

Figure 51a

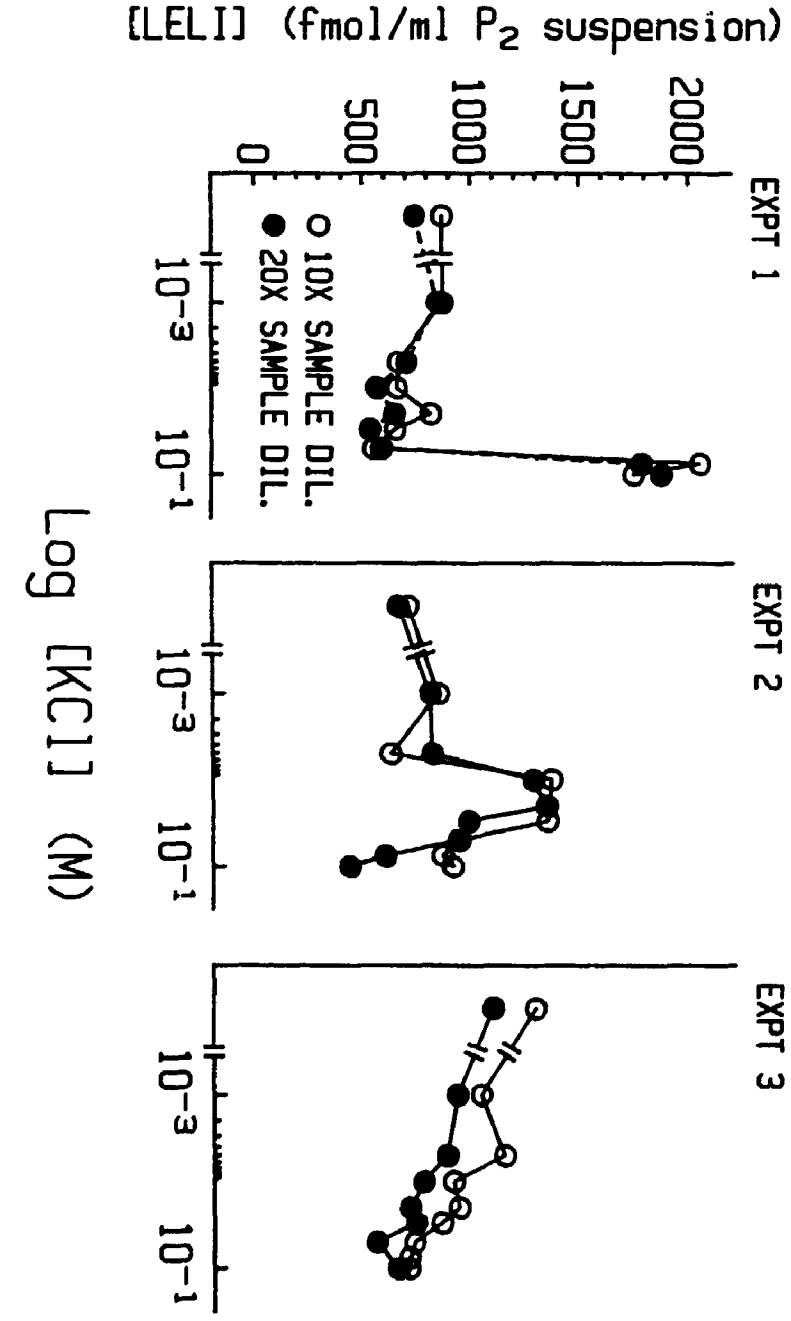

RELEASE OF LELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 5μM ATROPINE
[LELI] (fmol/ml P2 suspension)
0
500
1000
1500
2000
EXPT 1
EXPT 2
EXPT 3
O 10X SAMPLE DIL.
● 20X SAMPLE DIL.
10-3
10-1
Log [KCl] (M)

Figure 51b

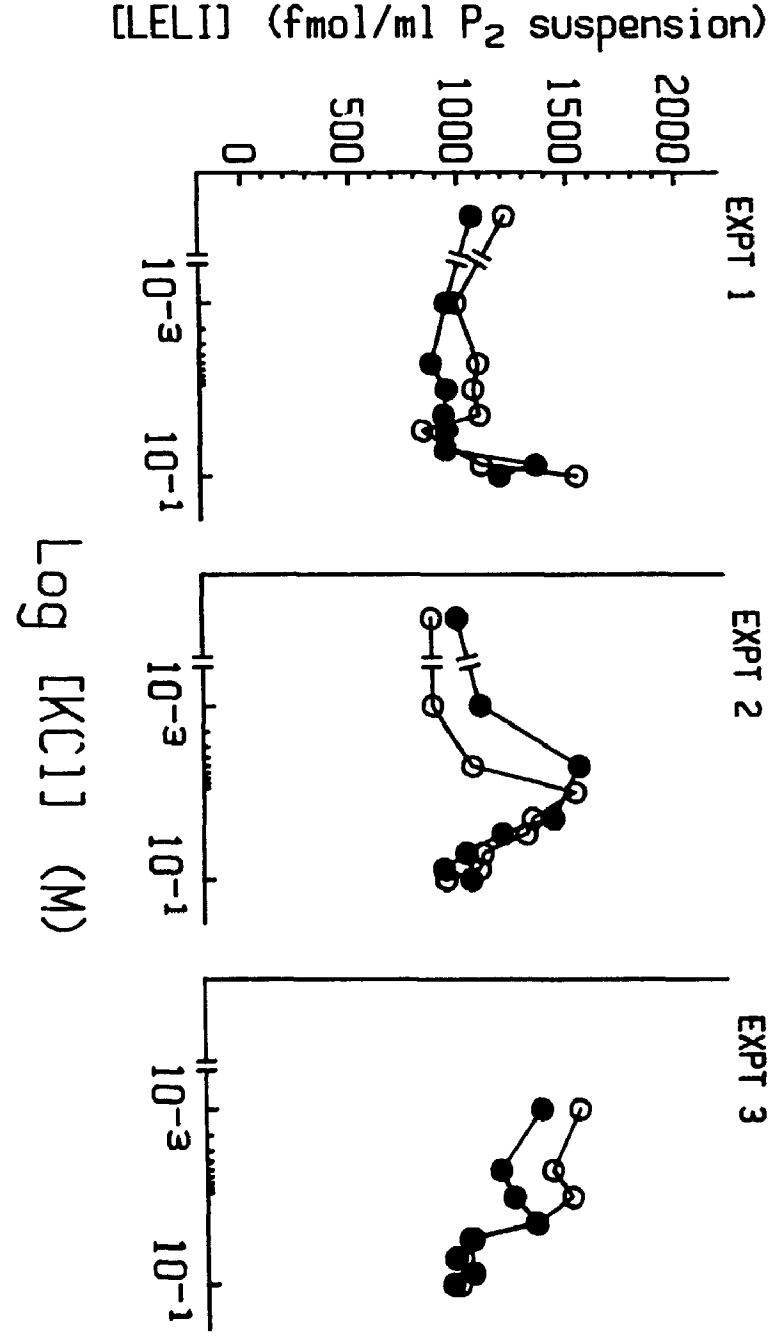

RELEASE OF LELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 20μM NALOXONE
[LELI] (fmol/ml P2 suspension)
0
500
1000
1500
2000
EXPT 1
EXPT 2
EXPT 3
10-3
10-1
Log [KCl] (M)

Figure 51c

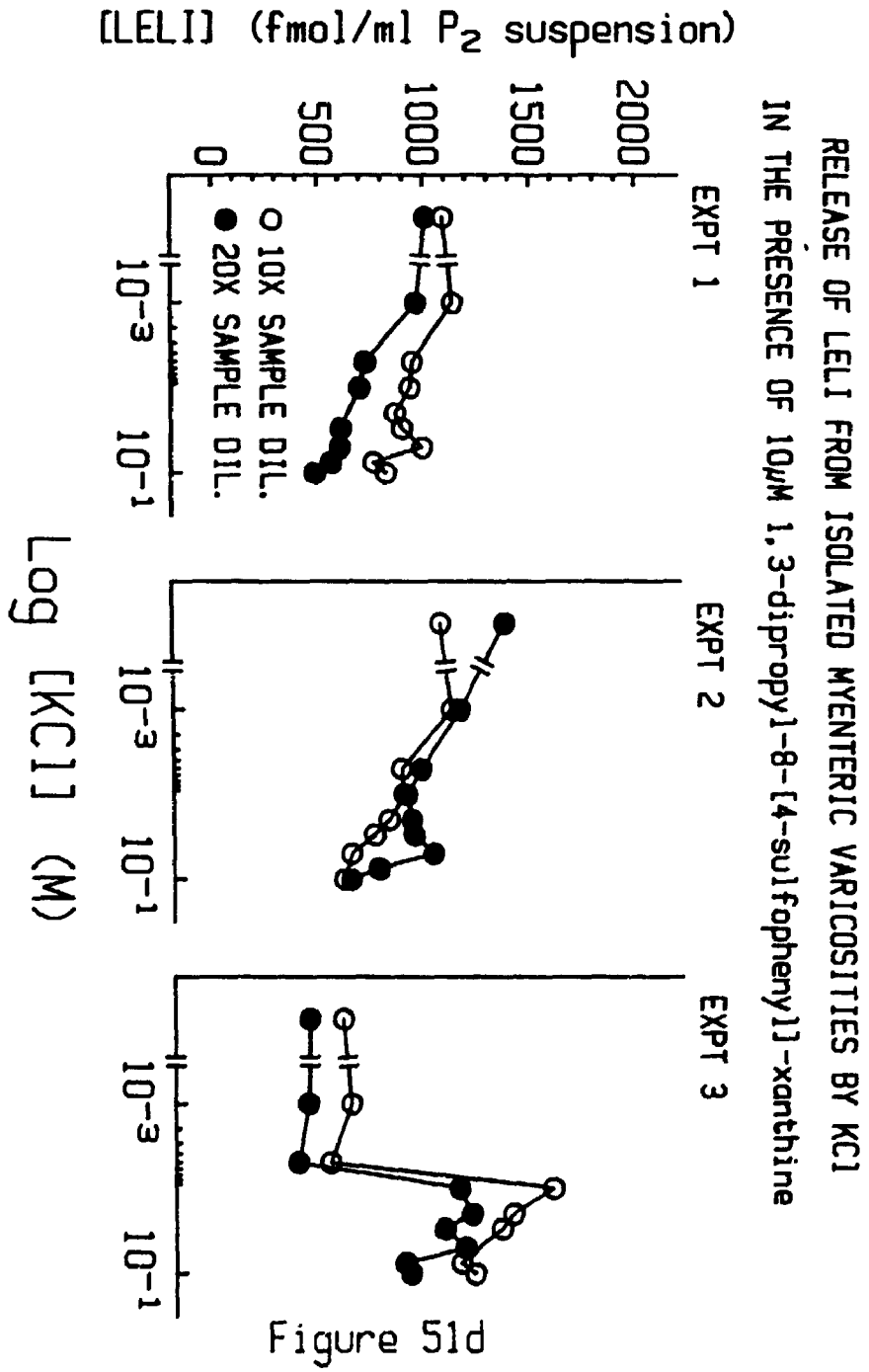

RELEASE OF LELI FROM ISOLATED MYENTERIC VARICOSITIES BY KCl
IN THE PRESENCE OF 10μM 1,3-dipropyl-8-[4-sulfophenyl]-xanthine
[LELI] (fmol/ml P2 suspension)
2000
1500
1000
500
0
EXPT 1
EXPT 2
EXPT 3
O 10X SAMPLE DIL.
● 20X SAMPLE DIL.
10-3
10-1
Log [KCl] (M)

Figure 51d

Fig. 52. Effect of the calcium channel antagonist methoxyverapamil (D600) on the basal release of a) SPLI, b) a-NKLI and c) LELI, in the presence of incrementing concentrations of calcium in the P_2 suspension. The P_2 was resuspended in calcium-free Locke's solution and allowed 60 min. equilibration at 37 C. Following initial incubation with bacitracin (5 min), 500 ul of P_2 suspension was added to 500 ul Lockes solution in the incubation tubes containing incrementing concentrations of calcium (reaction for 5 min), with or without D600. The reaction was stopped by centrifugation at 13,000 X g for 1 min. The supernatants were boiled for 7 min to inactivate enzymes. The results represent the findings from one experiment. Essentially identical results were obtained in one other experiment. Intra-assay variability is < 5%. Approximately 0.75 mg tissue used in each incubation.

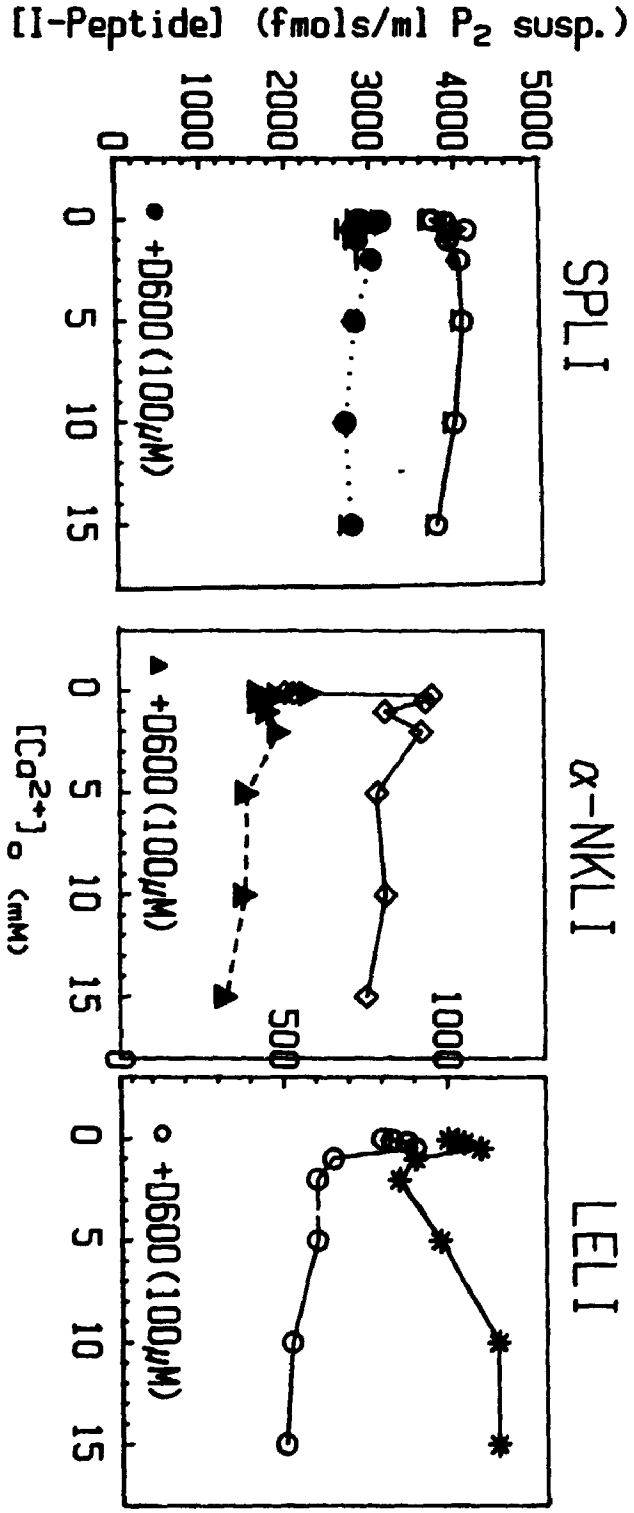

[I-Peptide] (fmols/ml P_2 susp.)
0
1000
2000
3000
4000
5000
a)
SPLI
+D600(100μM)
b)
α-NKLI
+D600(100μM)
500
1000
c)
LELI
+D600(100μM)
0
5
10
15
$[Ca^{2+}]_o$ (mM)

Figure 52

Fig. 53. Contractile responses of the atropinized guinea pig longitutinal muscle - myneteric plexus strip (LMMP) to GRP and bombesin. The tissue was bathed in Krebs' buffer (gassed with 95% O_2/5% CO_2) and equilbrated at 37 C for 45-60 min. before exposure to any drugs. The contractions, recorded isotonically, were expressed as the percent of the contraction obtained with 5nM CCK-8. Each bar represents the mean $\pm$ S.E.M. from 4 - 6 preparations. * the response is significantly different ($p < .05$) from that obtained with luM GRP alone. a) dose dependent contraction of the LMMP by GRP. b) Inhibition of GRP-mediated contraction by TTX and the substance P antagonist [d-Pro^4,d-trp^{7,9,10}] SP (4-11) (SP-A).

Figure 53

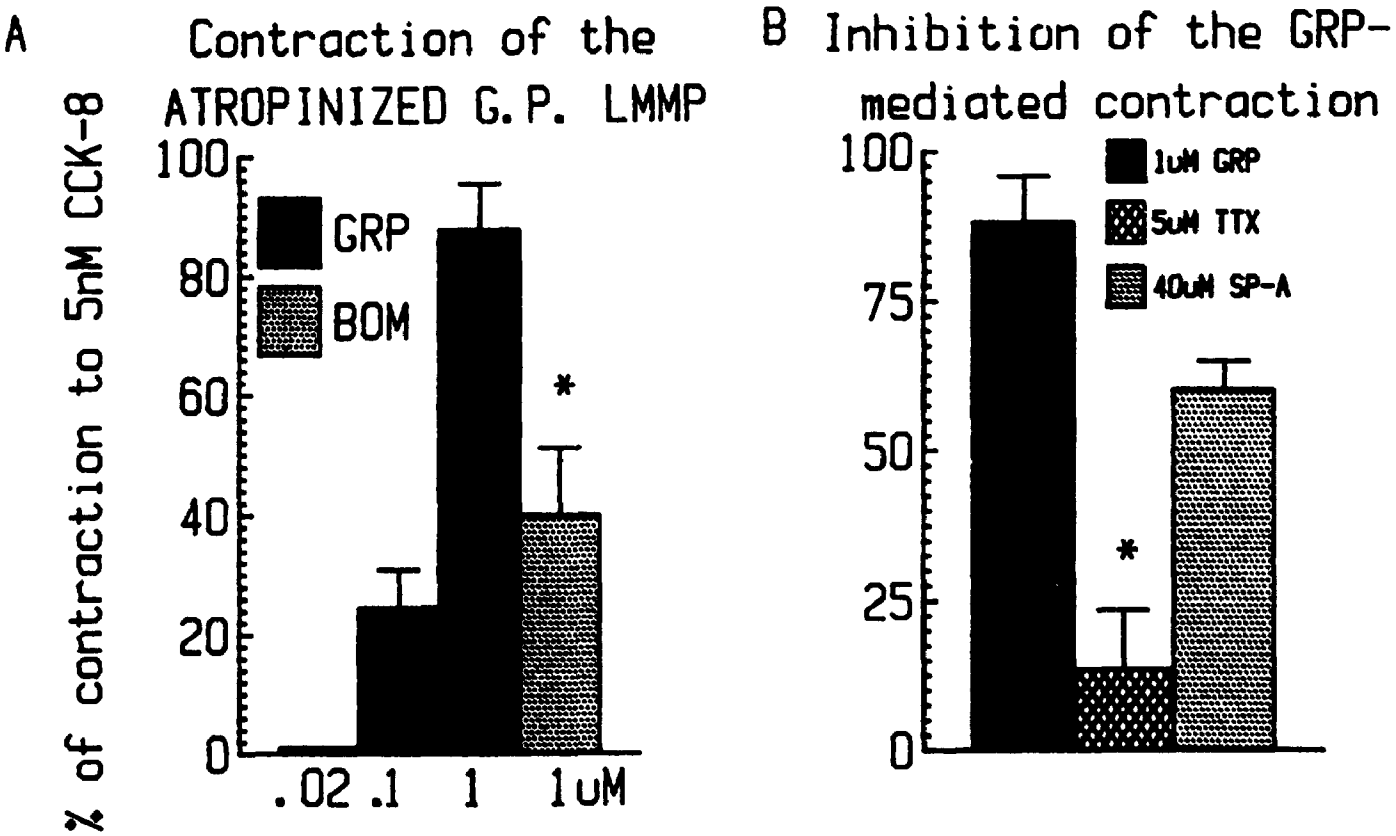

A
Contraction of the ATROPINIZED G.P. LMMP
% of contraction to 5nM CCK-8
100
80
60
40
20
0
GRP
BOM
*
.02
.1
1
1uM
B Inhibition of the GRP-mediated contraction
100
75
50
25
0
1uM GRP
5uM TTX
40uM SP-A
*

Fig. 54. Release of a) a-NKLI and b) SPLI from isolated myenteric varicosities (P_2) by bombesin and GRP. Following resuspension of the P_2 in Locke's solution (pH 7.2), a 1.0 h equilibration was allowed at 37 C. The P_2 was then incubated with bacitracin (5mM) for 7 min and then the 3 min. reaction was initiated by addition of 700 ul of P_2 suspension to the incubation tube containing incrementing concentrations of the P_2. The results shown are from a single experiment. Similar results were obtained from two additional experiments. Approximately 0.75 mg tissue used in each incubation.

Figure 54a

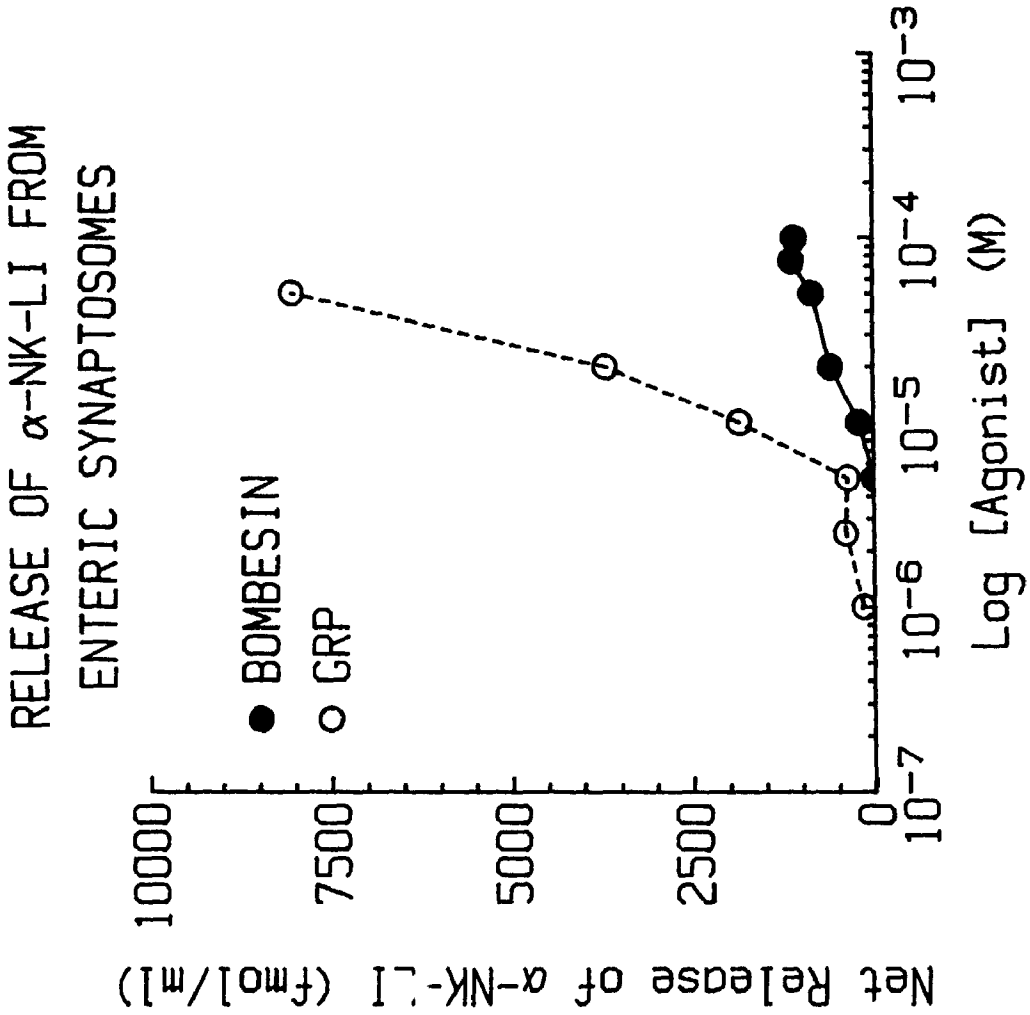

RELEASE OF α-NK-LI FROM
ENTERIC SYNAPTOSOMES
● BOMBESIN
○ GRP
Net Release of α-NK-LI (fmol/ml)
10000
7500
5000
2500
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
Log [Agonist] (M)

Figure 54b

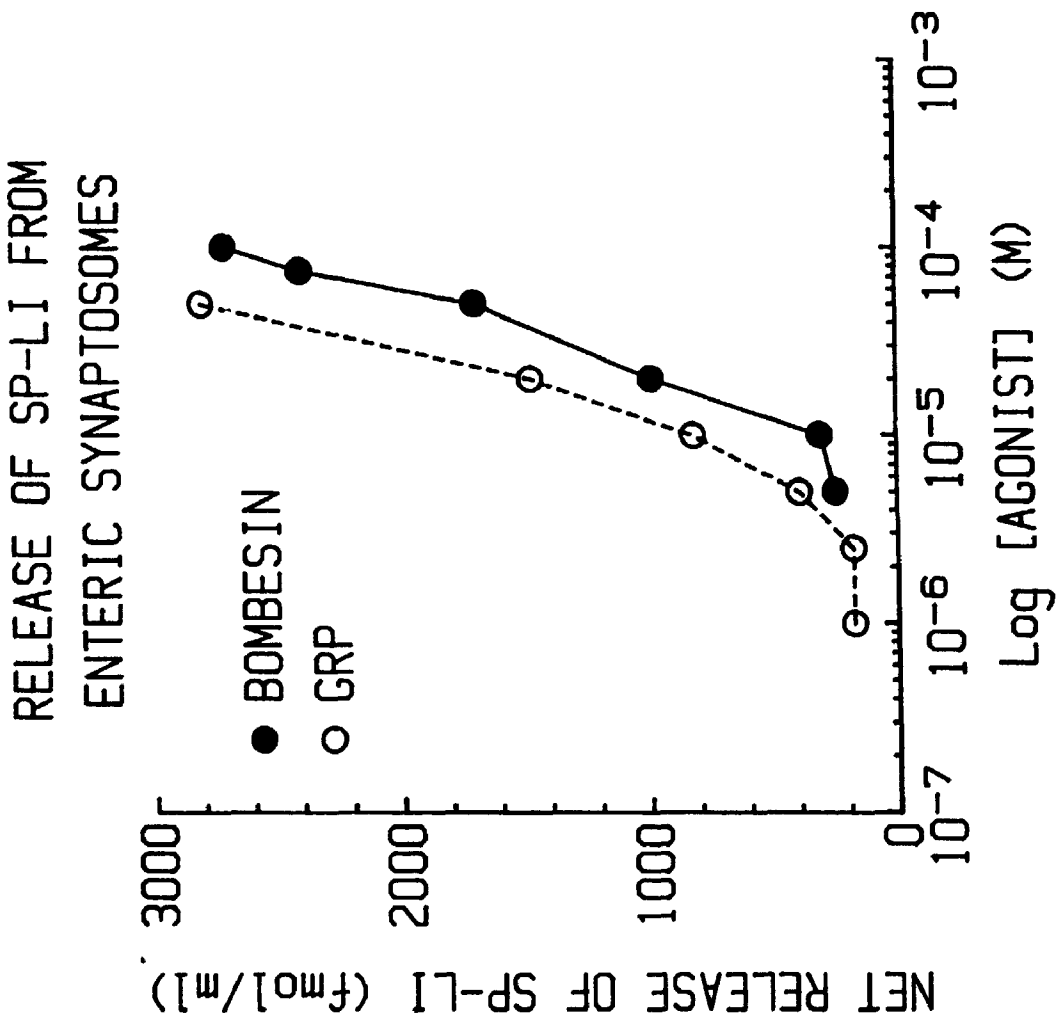

RELEASE OF SP-LI FROM
ENTERIC SYNAPTOSOMES
BOMBESIN
GRP
NET RELEASE OF SP-LI (fmol/ml)
3000
2000
1000
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
Log [AGONIST] (M)

Fig. 55a. Release of alpha-NKLI from isolated myenteric varicosities (P_2), following a 30 min. or a 60 min. equilibration at 37 C. The P_2 was resuspended in Locke's solution (pH 7.2). The results shown are from a single experiment. Approximately 0.75 mg tissue used in each incubation.

Figure 55a

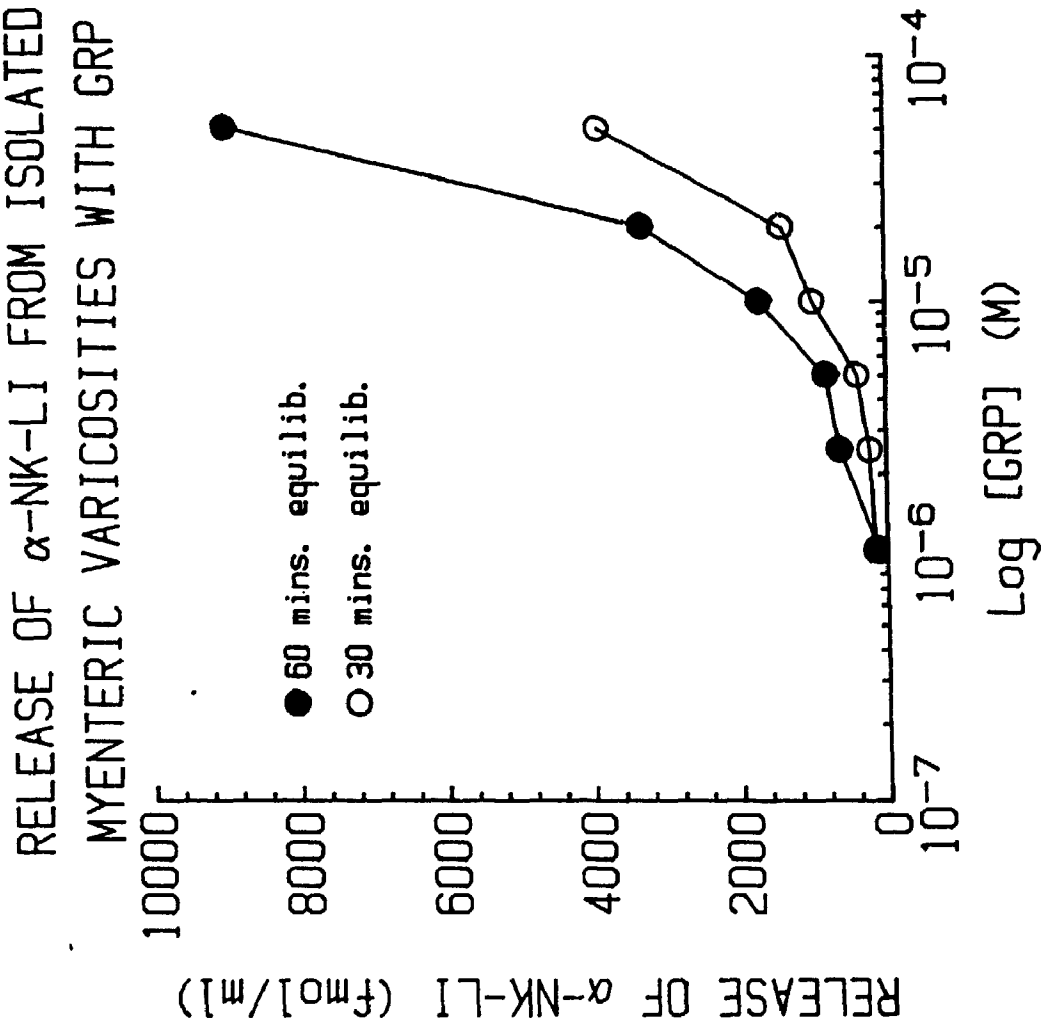

RELEASE OF α-NK-LI FROM ISOLATED
MYENTERIC VARICOSITIES WITH GRP
RELEASE OF α-NK-LI (fmol/ml)
10000
8000
6000
4000
2000
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Log [GRP] (M)
● 60 mins. equilib.
○ 30 mins. equilib.

Fig. 55b. Release of SPLI from isolated myenteric varicosities (P_2), following a 30 min. or a 60 min. equilibration at 37 C. The P_2 was resuspended in Locke's solution (pH 7.2). The results shown are from a single experiment. Approximately 0.75 mg tissue used in each incubation.

Figure 55b

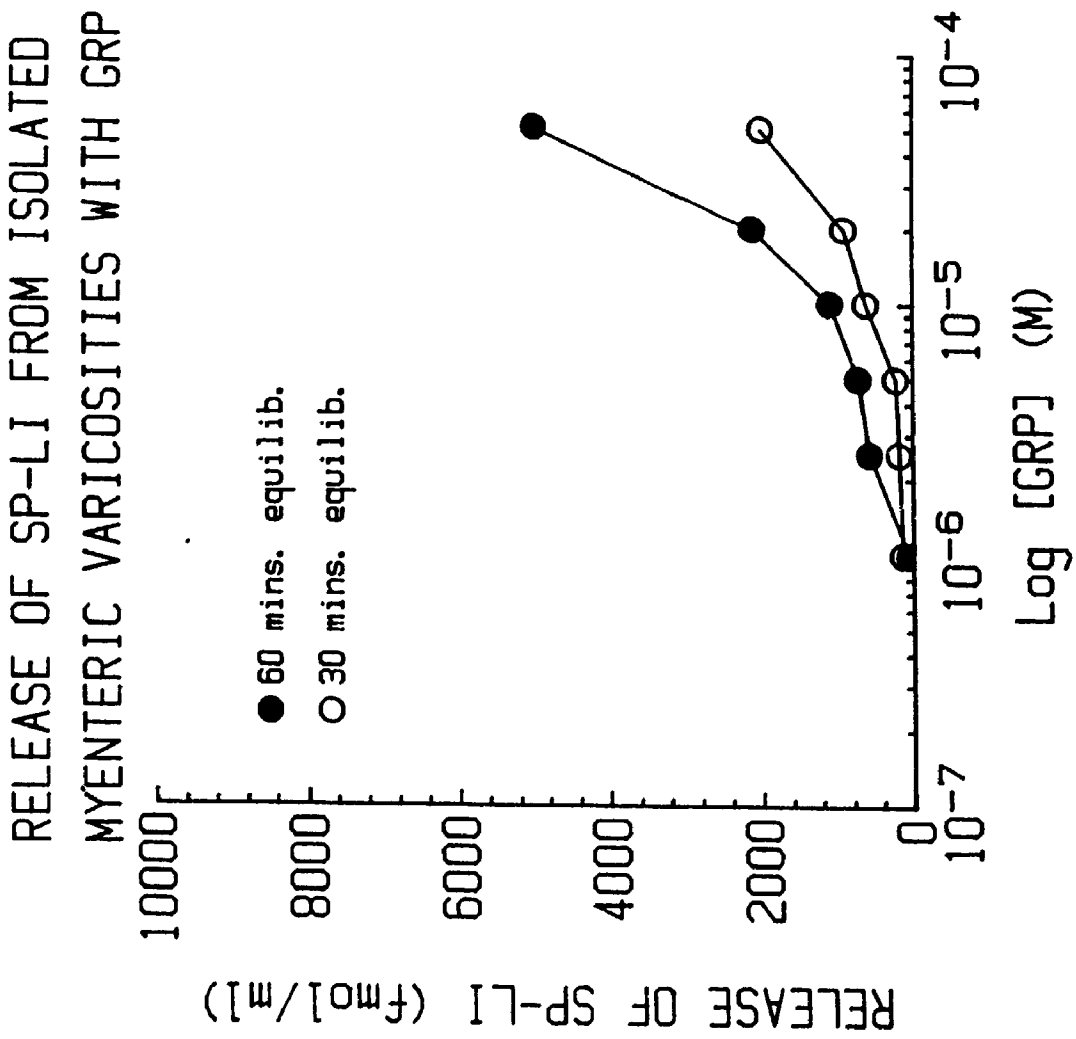
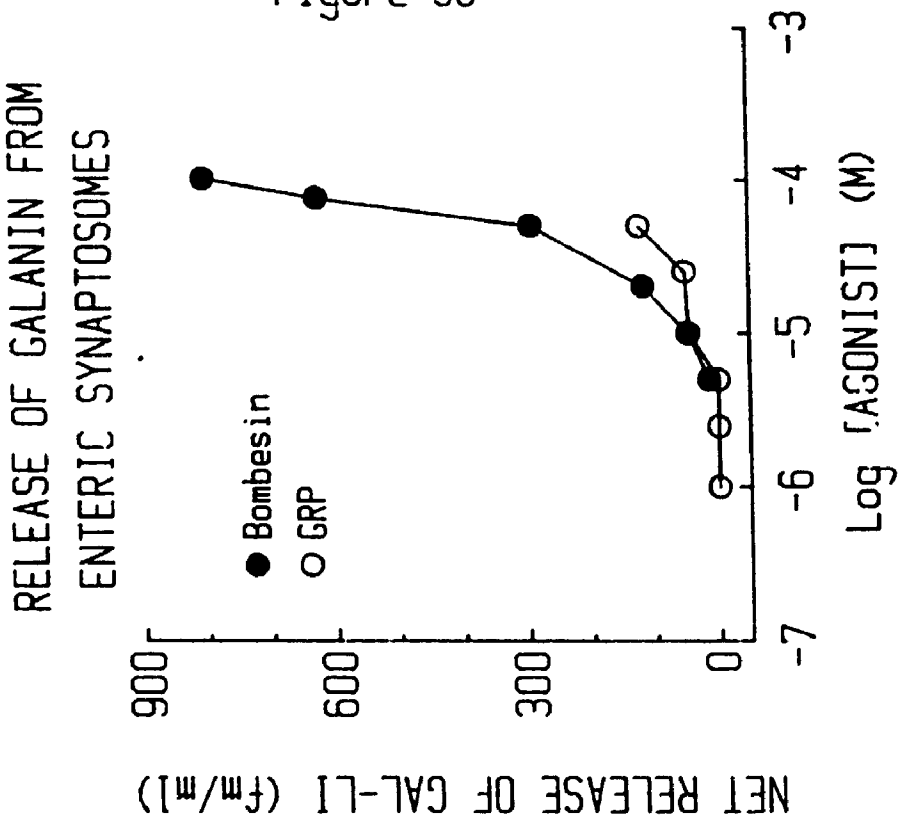

RELEASE OF SP-LI FROM ISOLATED
MYENTERIC VARICOSITIES WITH GRP
● 60 mins. equilib.
○ 30 mins. equilib.
RELEASE OF SP-LI (fmol/ml)
10000
8000
6000
4000
2000
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Log [GRP] (M)

Fig. 56. Release of galanin-like immunoreactivity from isolated myenteric nerve endings (P_2) by GRP and bombesin. The results are representative findings from one of three such experiments. The 3 minute release reaction was initiated following a 60 min equilibration of the P_2 in Locke's solution (pH 7.2) at 37 C and a pre-incubation of 7 min with bacitracin (5mM). Approximately 0.75 mg tissue was used in each incubation.

Figure 56


RELEASE OF GALANIN FROM
ENTERIC SYNAPTOSOMES
● Bombesin
○ GRP
NET RELEASE OF GAL-LI (fm/ml)
900
600
300
0
-7
-6
-5
-4
-3
Log [AGONIST] (M)

DISCUSSION

Neuromodulation of transmitter release from myenteric nerves of the small intestine is achieved through a number of different mechanisms whose involvement in enteric nerve function is still poorly understood. The term neuromodulation has been used loosely to describe several different kinds of phenomena. In the present discussion it will be defined in a more restricted way as the ability of certain agents to inhibit or stimulate the release of neurotransmitters from myenteric neurons. Neuromodulation of transmitter release leads to intracellular biochemical changes which result from an interaction of these agents with specific cell surface receptors present on myenteric nerve endings. Detailed discussion of the complex mechanisms which exist for transducing these extracellular signals into a change in transmitter release is beyond the scope of the thesis. It is sufficient to state, that both direct ligand-gated ion channels as well as indirect binding of a ligand to its receptor(s) triggering the formation of second messenger substances within a cell, may be involved in neuromodulation of transmitter release from myenteric nerve endings.

Many mechanisms exist which are involved in coordinating the integrative activity of myenteric neurons of the small intestine. Among these, heterogeneity of receptors, heterogeneity of neuronal

types, co-release of neuromediators and interactions between these released neuromediators are adoptations of the enteric nervous system which illustrate the complexity of the regulatory processes that may govern enteric nerve function. Neuromodulation of transmitter release from enteric neurons may ultimately affect the postjunctional smooth muscle of the small intestine, and neuromodulators themselves may have direct receptor mediated postjunctional effects on smooth muscle. The issue addressed in this thesis is characterization of adenosine receptors located on myenteric nerve endings, at which endogenous adenosine acts to modulate the release of transmitters. In a broader sense, the studies carried out addressed the issue of neuroregulation of transmitter release from myenteric neurons by nucleosides.

Originally, extracellularly located adenosine receptors were defined according to their inhibitory (A_1 or R_i) or stimulatory (A_2 or R_a) effects on adenylate cyclase. Both classes of receptor occur in brain, and both are antagonized by the methylxanthines. More recently however, the criteria for classification of adenosine receptor subtypes have been revised (Stone,1984) with the emphasis being on the potency differential between A_1 and A_2 receptor agonists, because the involvement of adenylate cyclase in the responses to adenosine was not always clear. Those tissues in which the potency of R-PIA and CHA > NECA are described as possessing A_1 receptors and those tissues in which this potency order is reversed display A_2 receptor characteristics. At present, the available

literature suggests that stereospecificity of a given response does not necessarily indicate mediation of a response by an A_1 receptor. (Fredholm et al,1982; Edvinsson and Fredholm, 1983).

In a recent review by Ribeiro & Sebastiao (1986), an A_3 xanthine-sensitive adenosine receptor was proposed which is not coupled to adenylate cyclase. It has the agonist profile R-PIA, CHA, NECA > CADO, and S-PIA is usually less potent than CADO. It is believed to be distributed mainly in excitable tissues. The A_3 receptor is linked to calcium influx, but it is not clear how adenosine affects calcium needed for transmitter release. It has been speculated by Ribeiro & Sebastiao (1986) that the A_3 adenosine receptor is the voltage-dependent calcium channel.

Binding studies carried out on broken-cell preparations of brain tissue have permitted the fundamental characteristics of affinity and density of binding sites to be determined for several nucleoside ligands (for references, see Daly,1983). In contrast, at the peripheral nervous system comparable information was lacking. Despite the extensive structure activity information obtained from functional studies using intact guinea pig ileum, the characteristics of the adenosine receptor(s) on enteric nerve endings (Gustaffson et al,1978; Okwuasaba et al,1978; Sawynok & Jhamandas,1976; Hayashi et al,1977; Cook et al,1979; Maguire & Satchell,1979; Paul et al,1982) were not known.

Isolated myenteric varicosities (autonomic synaptosomes) (PV) prepared from the guinea pig ileum were used to examine the characteristics of the binding sites which have affinity for nucleoside ligands. Myenteric nerve endings have been prepared by several laboratories and release of neuromediators from these entities has been demonstrated (Briggs & Cooper,1982; White & Leslie,1982; Reese & Cooper,1982). The choice of the PV as a substrate in ligand binding studies was based on the premise that intact varicosities contain the adenosine receptor(s) mediating the inhibition of transmitter release at the stimulated ileum preparation.

CHARACTERIZATION OF PARTIALLY PURIFIED MYENTERIC VARICOSITIES

Isolation of partially purified myenteric varicosities followed established, conventional methodology with minor modifications and yielded preparations that were clearly enriched with spontaneously resealed varicosities. Occluded LDH activity, measured as an index of cytoplasm surrounded by a cell membrane, yielded a mean value which was appreciably greater than that reported by White and Leslie (1982) for a similar preparation. This may be attributed to the use of younger animals in the present study. In general it was noted that animals weighing 200 to 300 g gave lower overall yields of PV (in terms of protein / guinea pig) but displayed higher occluded LDH activity per milligram of protein.

Increasing the enrichment of the preparation with isolated

varicosities, as measured by increasing occluded LDH activity, resulted in a related increase in specific binding of the radioligands. The obtaining of maxima in the levels of specific binding achieved, suggested that the PV preparations were adequately enriched with intact nerve endings for the performance of binding competition and saturation studies, since further enrichment of the PV with varicosities would not further increase the total specific binding of [^{3}H]-R-PIA and [^{3}H]-NECA.

Electron microscopic studies were carried out on aliquots of PV as another means of confirming the presence of intact varicosities and to determine the morphological nature of this tissue. Varicosities of various types were observed in the PV and these could be categorized according to the descriptions presented by Furness and Costa (1980). The presence of nerve endings of peptidergic, cholinergic, purinergic, and tryptaminergic character may be imputed from such diverse morphologies. The uptake of serotonin (Jonakait et al.,1979), norepinephrine and choline (Briggs and Cooper,1982) as well as the release of loaded labeled Ach (Briggs and Cooper,1982) by isolated myenteric varicosities has been demonstrated. These reports demonstrated the potential utility of the isolated varicosity preparation in functional studies. The presence of peptide containing nerve endings was verified in studies measuring the neuronal contents of several peptide immunoreactivities in lysates of PV. In these studies, SPLI, a-NKLI, VIPLI, GRPLI, MELI, LELI and GALLI were identified.

Clearly, the PV was also enriched with many different types of peptdidergic nerve endings, providing a heterogeneous substrate consisting of both peptidergic and cholinergic nerve endings for nucleoside binding studies. Functional studies involving endogenous neuropeptide release from such isolated myenteric varicosities and their regulation by nucleosides was also an appealing avenue for investigation.

SATURATION BINDING STUDIES WITH TRITIATED NUCLEOSIDE ANALOGS TO PV AND MITOCHONDRIAL FRACTIONS.

Initial characterization of the peripheral nucleoside binding sites involved determination of the saturation binding characteristics of the labeled A_1 adenosine receptor ligand CHA and the mixed A_1/A_2 ligand NECA at both the partially purified varicosity preparation and at mitochondrial fractions (MITF). In these studies, it was possible to detect saturable components for both [^{3}H]-CHA and [^{3}H]-NECA binding at PV. The binding isotherms yielded affinities which were closely related to the concentration range over which these adenosine analogs cause presynaptic inhibition of transmitter release from both cholinergic (Paton,1981; Paton & Webster,1984; Christofi & Cook,1987) and tachykinergic nerves (see below) present in the intact guinea pig ileum. The equilibrium dissociation constants (K_d) obtained for the radioligands at PV correlate well with their respective EC_{50} estimates obtained at the stimulated guinea pig ileum. Although not a necessity, such parallel findings, strongly support the contention that high-affinity binding sites for

CHA and NECA represent functional adenosine receptors. The low capacity of the respective binding sites on PV further supports this contention.

NECA, a mixed A_1/A_2 adenosine receptor ligand and CHA, an A_1-selective ligand share the same binding affinity (K_d) and label the same concentrations of adenosine recognition sites at PV. These data are consistent with the presence of atypical adenosine recognition sites, not classifiable according to the current criteria. If NECA and CHA are binding strictly as A_1 receptor ligands, the data is consistent with the presence of A_1 adenosine receptors on myenteric nerve endings, but, as will be addresed later, this may not be the case.

The Scatchard data for ^{3}H-[NECA] at PV best fit a one site model (n=3). In one experiment, the saturation data was also statistically fitted by a two site model, revealing a second low affinity, high capacity site. The precise parameters of this site could not be accurately determined because of increases in non-specific binding associated with increasing ligand concentration.

At this point it is not known to what extent this site represents the 8-phenyl-theophylline insensitive NECA binding sites revealed in auto-radiographic studies (Buckley & Burnstock,1983). The scatter in the data points used for Scatchard analysis is in part responsible for the inconsistent finding of two distinct sites. An impractically

large n value would have been required to determine with confidence if a second low affinity binding site for NECA exists. It is worth noting that curvilinearity of the Scatchard plot usually does not represent two distinct receptor binding sites (Cuatrecasas & Hollenberg,1976). Underestimation of the specific nonreceptor binding sites for [3H]-[NECA] may explain curvilinearity of the Scatchard plots, since cold NECA was used to estimate nonspecific binding of labeled NECA in these studies.

The saturation binding profile for ^{3}H-[NECA] at the MITF closely paralleled that obtained at the PV. The presence of similar high-affinity binding sites for NECA at both PV and MITF's is not surprising as similar results were obtained by Newman and co-workers (1980) using mitochondria and synaptosomes prepared from brains of several species. The data are consistent with the presence of A_1 adenosine recognition sites on subcellular components contained in the mitochondrial suspension. It is unlikely that the mitochondrial binding sites represent specific nonreceptor binding to macromolecules in the MITF, since these sites have identical binding characteristics with adenosine recognition sites present on nerve varicosities.

Contamination of MITF with nerve varicosities may explain the binding of [^{3}H]NECA to the MITF although non-specific binding to this fraction undoubtedly occurs. In support of this contention, studies on the distribution of neuropeptide-like immunoreactivities

on sucrose gradients, showed that some intact myenteric varicosities co-distribute with mitochondria at the 1.2M sucrose density layer (White,1982).

In contrast to the findings obtained with [^{3}H]-CHA at PV, the binding of this radioligand to rat brain synaptosomal membranes revealed two binding sites with K_d values of 0.7nM for the high affinity binding site and 2.4nM for the low affinity site. The respective Bmax values were 230 and 120 fmol/mg protein. Clear differences in Bmax and K_d values between the central and peripheral binding sites may indicate that the peripheral adenosine receptor(s) represent a novel class of adenosine receptors distinct from the A_1 and A_2 adenosine receptor types described in brain membranes. However, species differences in nucleoside binding sites may also have contributed to the differences observed. An additional factor which must be considered is the difference in the nature of the preparations used in these studies. The present studies used intact synaptosomes in contrast to the use of synaptosomal membranes in the CNS work. The exposure of additional binding sites may have contributed to these differences. The observation by Williams and Valentine (1985) of higher affinity binding sites for CHA obtained at crude myenteric plexus synaptosomal membranes supports this possibility.

Possible heterogeneity of adenosine receptors which are located on isolated myenteric varicosities of guinea pig small intestine.

Considerable evidence suggests that heterogeneity of adenosine receptors exists in the central nervous system. In addition to its binding to A_1 and A_2 adenosine receptors (Yeung and Green,1983), [^{3}H]-NECA may be involved in the binding to subtypes of the A_2 receptor (Bruns et al, 1986) in the CNS. On brain membranes, there is also considerable evidence for heterogeneity of A_1 adenosine binding sites (Murphy and Snyder,1982). It now appears that subclasses of stimulatory A_2 adenosine receptors linked to adenylate cyclase exist in the CNS (Londos et al,1983; Daly et al,1983; Premont et al,1979; Premont et al,1983). [^{3}H]NECA binds to A_1 adenosine receptors in cerebral cortical and hippocampal membranes (Yeung and Green, 1983b). In striatal membranes, this ligand binds to both A_1 receptors and the high-affinity A_2 receptors (Yeung and Green,1983b).

Clearly heterogeneity of adenosine receptors had been shown to exist in the central nervous system. Comparable information at the myenteric nervous system was lacking. The possible existence of multiple adenosine receptors at the peripheral locus was thus investigated using the PV as a suitable substrate in competition studies.

Ligand competition experiments, using [^{3}H]-CHA, [^{3}H]-R-PIA and [^{3}H]-

NECA as the labeled ligands, allowed the binding characteristics of the adenosine receptor(s) on myenteric nerve endings to be further examined. The competition displayed by R-PIA, CHA and NECA for the binding of equimolar concentrations of their labeled counterparts did not allow any distinction to be made between the binding sites labeled by these ligands. The results show similar competition profiles and IC_{50} values for all three ligands and did not permit the subclassification of the binding sites on PV into A_1 or A_2 adenosine recognition sites, according to current criteria. Furthermore, the displacement of [^{3}H]-CHA by unlabeled NECA generated similar displacement profiles with comparable IC_{50} values (ie. CHA = NECA in displacing [^{3}H]-CHA). In contrast, the displacement profiles obtained using [^{3}H]-NECA and the A_1 ligands 2CADO, CHA, R-PIA, CPA and R-PBA as displacers, revealed a clear distinction in binding characteristics. The A_1 ligands were poor competitors for the sites on myenteric nerve endings labeled by NECA, except for 2CADO. The potency order of selective adenosine analogs as displacers of [^{3}H]-NECA was NECA = 2CADO > R-PIA = CHA = CPA = R-PBA. This profile is different from that obtained for cAMP accumulation in brain slices and homogenates (ie. NECA > 2CADO > R-PIA) arguing against a link between peripheral adenosine receptors and adenylate cyclase activation (Daly,1983). The present findings support the existence of two distinct binding sites at this locus. It appears that one binding site recognizes both A1 receptor ligands like CHA as well as NECA while the other binding site recognizes NECA and 2CADO only. The ability of 2CADO to displace the residual

binding of [^{3}H]-NECA not recognized by A_1-selective ligands, suggests that such binding may represent specific binding to an A_2 type adenosine receptor. Such heterogeneity of binding sites revealed with [^{3}H]-NECA is consistent with autoradiographic data obtained using the myenteric plexus of the guinea-pig ileum (Buckley and Burnstock, 1984).

At the CNS Reddington et al (1985) demonstrated heterogeneity of binding sites on rat cortex and striatum using an approach similar to that used in the present studies. In that report the differential binding profiles of R-PIA and NECA over a range of ligand concentrations, determined using labeled NECA, was used to indicate the presence of both A_1 and A_2 receptors. The presence of A_1 receptors was inferred from the higher potency of R-PIA as a displacer of the label compared to that of NECA. At higher concentrations the reverse potency order indicated to those investigators the presence of an A_2 adenosine receptor site labeled by NECA. Although the present results confirmed this heterogeneity of adenosine binding sites for enteric nerves, they did not permit any resolution into A_1 and A_2 sub-types in an analogous manner, since no significant difference in potency of these displacers was detected at lower ligand concentrations.

The competition curves generated using the A_1 selective adenosine receptor antagonist DPSPX as the unlabeled displacer showed a clear difference between CHA and NECA binding. It is evident that DPSPX is

a more effective displacer of the binding of labeled CHA than it is of labeled NECA. Since DPSPX displaces all of the binding labeled by the A_1 ligand CHA, while only displacing a portion of that labeled by NECA, it appear that DPSPX displaces that portion of the binding of NECA which labels the A_1 site. These data are consistent with the 3.4 fold selectivity for the A_1 site which has been reported for this compound (Daly, 1985). The findings confirm and extend the results obtained from the competition data established using the agonist ligands analysis. Thus, heterogeneity of binding sites, and possibly of functional receptor sites in the enteric nervous system is supported by the present findings. On this basis, it is clear that the similarity between the equilibrium dissociation constants (K_d) obtained for [^{3}H]-NECA and -CHA at PV cannot be explained by suggesting that these radioligands are binding strictly as A_1 adenosine receptor ligands.

The selectivity of DPSPX, although greater than that of theophylline, may not have been sufficient to clearly resolve the binding into distinct subtypes of adenosine receptor sites on myenteric nerve endings. The synthesis of additional selective and soluble adenosine receptor antagonists, as well as more selective A_2 agonists, should permit the resolution of this question. Recent evaluation of the binding characteristics of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) to membrane preparations showed that it was 500-fold more effective at A_1 than at A_2 sites (Lee & Reddington,1986), suggesting that it may be of considerable use in

future studies on heterogeneity of adenosine receptors at the myenteric locus.

The A_1-insensitive component of the specific binding of NECA to PV seen in competition experiments (Williams & Valentine, 1985; Christofi & Cook,1987) most likely corresponds to the A_1 ligand-insensitive sites for NECA at PV revealed in saturation binding studies. The 8-phenyltheophylline-insensitive binding of NECA to myenteric neurons reported by Burkley & Burnstock (1984) is unlikely to correspond to such sites, since 2CADO was able to displace all the specific binding of [^{3}H]-NECA to PV.

It is a distinct possibility that the binding site at PV which is insensitive to A_1-selective ligands represents the putative postjunctional or prejunctional A_2 adenosine receptor which has affinity for NECA and not selective A_1 ligands (Gustafsson et al,1985). Together with the saturation binding data, these findings from competition experiments are consistent with the presence of A_2 adenosine receptors in the PV.

Although heterogeneity of adenosine receptors is one explanation which is consistent with the ligand binding studies, other explanations must not be discounted. NECA may also have affinity for glial membranes, interstitial cells of Cahal, fragments of resealed membranes from ganglion cell bodies and mitochondrial membranes contaminating the PV. In most instances distinction of

specific receptor binding sites from specific nonreceptor sites is rather difficult if not impossible. The involvement of adenosine uptake sites, which themselves are heterogeneous as demonstrated by labeled dipyridamole binding (Marangos et al,1985), must similarly be considered in the interpretation of the binding data.

Patel et al (1982) showed in subcellular distribution studies of [^{3}H]-CHA binding to rat forebrain, that about 20% of the total binding is to microsomes, myelin and mitochondria, components present in the crude ileal membrane preparation. Binding of [^{3}H]-adenosine to subcellular components other than synaptosomal was observed by Newman et al (1981) at guinea pig cerebral cortical tissue. Similar studies have not been carried out with NECA, but it is reasonable to assume that a portion of the NECA binding is to such subcellular components. It must be remembered, however, that no smooth muscle was present in these CNS preparations unlike the PV. Thus, the A_2 adenosine receptor binding on smooth muscle may represent a relatively larger portion of the specific binding of NECA to PV than that binding which is expected to occur to other subcellular components in CNS preparations.

Ligand competition experiments were carried out following an incubation time of 12 min. The relatively rapid kinetics of the response to CHA and NECA in functional studies provided the rationale for this equilibration time. If NECA and the A_1 adenosine receptor ligands require different equilibration times greater than 12 minutes, then inadequate equilibration of A_1 ligands with binding

sites for [^{3}H]-NECA may also explain the inability of A_1 ligands to displace all of the specific binding of NECA to PV. It is unlikely that this explains the heterogeneity of binding sites at PV, since an A_1 insensitive component of binding of labeled NECA to PV, was also revealed in ligand saturation studies, which were carried out following a 2.0h equilibration time following resuspension of the synaptosomes.

Heterogeneity of the adenosine recognition sites on myenteric nerve endings may further be characterized by the following ligand-binding studies using both PV and MITF's.

i) Saturation binding isotherms for ^{3}H-[THEO] or -[DPSPX] in the presence or absence of selective adenosine receptor agonists

ii)Binding studies involving displacement of labeled theophylline from both PV and MITF by both A_1 and A_2 adenosine receptor agonists.

iii) Displacement of ^{3}H-[NECA] from PV in the presence of 50nM CPA, with various cold adenosine receptor antagonists.

iv) Binding isotherms for [^{3}H]-[NECA] and -[CHA] repeated in the presence of NBMPR photoaffinity label which irreversibly binds to high affinity adenosine receptor uptake sites.

v) Displacement of [^{3}H]NECA from PV in the presence of DPCPX, with various cold adenosine receptor antagonists.

<u>Affinity of various purine nucleosides for adenosine receptors on purified myenteric varicosities compared to their efficacy as presynaptic inhibitors of Ach release</u>.

The present studies have shown that specific nucleoside binding sites exist on myenteric nerve endings of the small intestine. The fundamental characteristics of these binding sites were obtained from ligand saturation experiments and ligand competition experiments. These studies revealed the presence of a heterogeneous population of nucleoside binding sites at PV which may correspond to biologically relevant adenosine receptor sites present on intact guinea pig ileal myenteric nerve endings (Okwuasaba et al,1978; Sawynok & Jhamandas, 1976; Hayashi et al,1977; Cook et al,1979; Maguire and Satchell, 1979; Paul et al,1982). A link between the binding affinity of nucleoside analogs obtained at PV and their biological efficacy as inhibitors of Ach release obtained at the stimulated ileum would provide strong support for the contention that these binding sites represent the functional adenosine receptors present on intact myenteric nerve endings. This provided the rationale for further ligand binding studies using PV.

Competition experiments using [^{3}H]-R-PIA, [^{3}H]-CHA and [^{3}H]-NECA revealed the relative affinities of several unlabeled nucleoside analogs and antagonists for binding sites at PV. Table 1 and figures 10a and 10b show the parallel between the effectiveness (IC_{50}) of these nucleosides as displacers of [^{3}H]-PIA and [^{3}H]-NECA and their inhibitory potency (EC_{50}) in the biological assay system (stimulated ileum). Clearly, the weak agonists had lower affinity for the binding sites on myenteric varicosities than did those of

intermediate or higher potency. The more potent agonist 2-chloroadenosine was as effective as unlabeled R-PIA and NECA as both a competitor for the binding sites and as an agonist in the functional assay.

The correlation between the binding affinities and the biological activities demonstrated here provides strong evidence for the comparability of the binding sites on PV with the adenosine receptor(s) located on cholinergic myenteric nerve endings. It is worth noting that higher affinity for a binding site and higher efficacy at a receptor are not necessarily related properties. However, the present correlation is highly suggestive of a parallel between the binding affinity of nucleosides on PV and their efficacy as inhibitors of Ach release at the stimulated ileum. These novel findings indicate that [^{3}H]-R-PIA and [^{3}H]-NECA are suitable ligands for identifying adenosine receptors in the peripheral nervous system. Previous studies using [^{3}H]-NECA were less successful in characterizing adenosine receptor sites in peripheral tissues (Schutz et al, 1982; Huttemann et al, 1984; Bruns et al,1986).

Inhibitory actions of adenosine and its analogs on neurotransmitter release have been described at a wide variety of neurons both centrally and peripherally (Phillis and Wu,1983; Fredholm et al.,1983). Thus, the presence of presynaptic adenosine receptors may be inferred on cholinergic, noradrenergic, dopaminergic,

tryptaminergic, GABAergic and peptidergic nerve endings, all of which are present in the PV. In the present work, it is unlikely that radioligand binding occurred selectively to any one type of varicosity. It is more probable that binding at many different types of varicosities present in the preparation occurred. The finding of unit slopes in the correlation between biological efficacy and binding affinity for both labeled NECA and labeled R-PIA, may support the presence of similar adenosine receptors at these different myenteric nerve endings and reinforces the probability of a physiologically relevant neuroregulatory role for nucleosides.

Other ligand competition experiments also served to reinforce the probable correspondence of the binding site(s) on PV identified by the radioligands, with the adenosine receptor site(s) present on myenteric nerve endings. The parallel between the similar efficacies of the nucleoside analogs R-PIA, CHA and NECA obtained in the bioassay system (EC_{50}) and their similar affinities for the binding site(s) (IC_{50}) labeled by their tritiated counterparts (Figure 13), provides support for this conclusion.

Similar IC_{50} values obtained for the displacement of equal concentrations of [^{3}H]-R-PIA and [^{3}H]-NECA (20nM) by the adenosine receptor antagonist theophylline parallels the similarity between the pA_2 values obtained for this antagonist in the bioassay (Christofi and Cook,1985). Furthermore, the higher potency displayed

by DPSPX in comparison to theophylline, which is reflected in the pA_2 values determined for these antagonists, is paralleled by the higher potency (IC_{50}) of DPSPX as a displacer of the A_1 ligand [^{3}H]-CHA in comparison to theophylline (Christofi and Cook, 1986). These similarities further confirm the presence of adenosine receptors on isolated myenteric varicosities which are involved in the neuroregulation of the release of both Ach and TK's.

The total binding of labeled NECA to broken membranes prepared from PV by sonication, was greater than that observed with intact PV. Enhanced availability of internal binding sites present in varicosities as well as increased surface area available for binding, may, in part, account for the present findings. Boiling the PV abolished the specific binding of NECA, which is consistent with the macromolecular, and thus putative receptor nature, of the specific binding of NECA. Binding of NECA probably occurs to an intact cell membrane protein structure which, when denatured by boiling, loses its affinity for NECA.

In contrast to the findings with NECA, boiling enhances total binding of labeled R-PIA but does not abolish its specific binding. The lipophilicity of R-PIA mentioned above may explain such nonspecific displaceable binding of R-PIA to PV. Therefore, binding data obtained with R-PIA must be interpreted with some caution. It appears that NECA may be a better ligand than R-PIA for identifying adenosine receptor site(s) on PV.

CHARACTERIZATION OF BIOLOGICALLY RELEVANT ADENOSINE RECEPTORS WHICH MEDIATE INHIBITION OF CHOLINERGIC RESPONSES OF THE STIMULATED GUINEA PIG ILEUM PREPARATION

The stable nucleoside analogs NECA, R-PIA, CADO and CHA yielded the same direction of response as well as the same potency as inhibitors of Ach release at the stimulated ileum (.2Hz,1msec). All stable analogs were more potent than adenosine (EC_{50}= 10^{-6}M) and S-PIA was less potent than NECA, R-PIA, CADO and CHA but more potent than ADO. These results with the exception of the difference between the diastereomers of PIA, are in close agreement with those reported by other investigators (Paton,1981; Bhalla et al,1984; Gustafsson et al,1985) at the the same preparation. This rank order of potency differs from the initial description of A_1 (R_i) receptors according to which R-PIA is more potent than adenosine, in turn being more potent than NECA at the A_1 receptors (Londos et al.,1980). Nor is it consistent with the revised criteria for subclassification of adenosine receptors into A_1 or A_2 types described by Stone(1984). It has been suggested however, that NECA and R-PIA might be equieffective at some A_1 receptors (Paton,1981; Collis,1983), in agreement with the present data. However, in the original report by Paton (1981), the presynaptic receptor in peripheral cholinergic nerve endings was defined as A_1 even though the carboxamide NCPCA was clearly more potent than R-PIA.

A notable exception to this rank order of potencies is the selective A_1 agonist CPA, which is clearly more potent at both the peripheral and the central adenosine receptors (Moos et al,1985). The A_1 adenosine receptor agonist N^6-R-1-phenyl-2-butyladenosine is equipotent to CPA as an inhibitor of Ach release from cholinergic nerve endings. The reportedly more selective A_1 agonist MCPA (Olsson, personal communication) exhibited a slight, but significantly lower potency than CHA or R-PIA. The structure activity profile of nucleoside agonists at the stimulated ileum is: CPA = R-PBA > NECA = CADO = R-PIA = CHA >/ MCPA > S-PIA > ADO. Ligand binding studies demonstrated a rank order of potencies of adenosine receptor ligands (ie. as displacers of the binding to PV by their tritium labeled counterparts) of R-PIA = CHA = NECA. This potency profile was consistent with that obtained in the functional studies and served to reinforce the utility of the PV preparation in characterizing peripheral adenosine receptors. This potency profile does not permit the subclassification of the adenosine receptor(s) on myenteric nerve terminals into A_1 or A_2 subtypes, on the basis of structure activity profiles obtained either at PV or at the stimulated ileum preparation.

In the lower concentration range, 10^{-9}M - 10^{-7}M, R-PIA was shown to be 8-fold more potent than S-PIA as inhibitor of contractile responses to nerve stimulation. The potency difference between the R and S-isomers of N^6-phenylisopropyladenosine is consistent with the presence of an A_2 adenosine receptor on cholinergic nerve endings.

In contrast, the potency difference between the diastereomers of PBA is suggestive of an A_1 adenosine receptor interaction.

The potency difference shown to exist between R- and S-PIA in the present studies is not in agreement with similar studies carried out by Gustafsson and co-workers (1985). They showed that R-PIA was at least 50-100 fold more potent than S-PIA at inhibiting the contractile responses to transmural nerve stimulation (see also Paton,1981), consistent with interaction at an A_1-like receptor mediating cholinergic inhibition. This discrepancy is difficult to reconcile. The difference in the present studies is unlikely to be a result of some direct smooth muscle stimulation, since the stimulated responses (.2Hz,1msec) were totally abolished by TTX, and since R-and S-PIA were shown by Gustaffson (1985) to have no effect on direct muscle stimulation in tissues stimulated at the same pulse duration as that used here. It is worth noting that the frequency of stimulation employed in the present studies is different than that used by others (Gustafsson, 1985; Paton,1981). With variation in frequency of stimulation, it may be speculated that recruitment of a subpopulation of myenteric cholinergic neurons, which display a higher threshold for excitation, may occur. The release of excitatory mediators from these neurons may be regulated by a different adenosine receptor than that which regulates cholinergic output from enteric nerve terminals at the lower frequency. If stereospecific differences for R/S PIA exist at the two receptors, this would explain the lower apparent difference between the isomers

of PIA in the present studies. Determination of the potency difference between the isomers of PIA at varying frequencies of stimulation would provide some insight here. Differences in the kinetics of the responses to the isomers may explain the discrepancy, if significantly different drug exposure periods were used previously in comparison to the present studies.

The lack of difference between the potencies of NECA, CHA and R-PIA and the lower potency of S-PIA in comparison to CADO are suggestive of the presence of an A_3 adenosine receptor (Ribeiro & Sebastiao, 1986) on myenteric nerve endings regulating Ach release. This receptor is believed to be present on nerve endings and adenosine acts by inhibiting calcium influx to block transmitter release. The only difficulty with this categorization of the peripheral adenosine receptor(s) is the similar potency of CADO with other A_1-selective adenosine receptor analogs. Perhaps it would be more appropriate to describe this receptor as an A_3-like adenosine receptor. Clearly, the peripheral adenosine receptor(s) is atypical in nature or the criteria for defining such receptors are inappropriate.

Lack of difference in the biological efficacies of the stable nucleoside analogs can not be explained by differences in the physical properties of these drugs. Both R-PIA and CHA are highly lipid soluble with a rather low solubility in aqueous media in comparison to the carboxamide NECA. Thus, when applied in the bath, these compounds may be sequestered into the phospholipid membranes

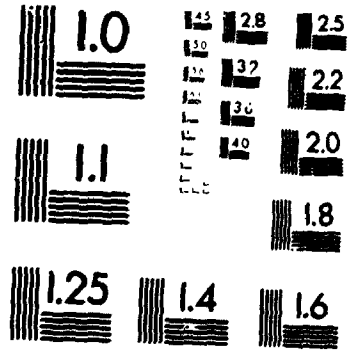
in which the receptor is embedded and, depending on their membrane / buffer partition coefficients, may achieve levels in the membrane an order of magnitude or more greater than those in the aqueous phase (Phillis & Barraco,1985). Therefore, during prolonged exposure to CHA and R-PIA (> 20 min.) both agents may become more concentrated in the immediate vicinity of the receptor than NECA (ie. both in ligand binding studies and functional studies), leading to an erroneous estimation of their potency with respect to less lipid-soluble analogs such as NECA. Sequestration and retention in the membrane may also explain the long duration of action of R-PIA and CHA and their long dissociation time in binding experiments in comparison with NECA ($t_{1/2}$ for CHA and PIA, 20 min at 25-37 C, for NECA < 20 sec at 0 C) (Schwabe,1983). Thus, the use of the standard pharmacological procedure of allowing responses to stabilize may be inappropriate when drugs of differing lipid solubility are being compared. In the only report in which A_1- and A_2-selective ligands have been tested on evoked responses in cerebellar and hippocampal slice preparations (Dunwiddie et al.,1983) it is clear that with brief applications in perfusion experiments NECA is more potent and that after prolonged application R-PIA becomes more effective. Since the stimulated ileum was exposed to the agonists (NECA, CHA and R-PIA) only for a relatively brief (3 - 5 min) time, it is difficult to reconcile the lack of difference between the potencies of NECA, R-PIA and CHA observed in the present studies, on the basis of their physical properties. Rather, the lack of difference may reflect the atypical nature of the peripheral adenosine receptor(s) on the basis

of A_1/A_2 classification criteria and may suggest the presence of an A_3 adenosine receptor.

Possible heterogeneity of adenosine receptors revealed in functional studies using the intact guinea pig ileum preparation.

The excellent correlation between binding affinity (at PV) and biological efficacy (stimulated ileum) obtained for several nucleosides and adenosine receptor antagonists indicated that the isolated myenteric varicosity contains the adenosine receptor responsible for the observed biological activity. From ligand binding studies, evidence was obtained supporting the heterogeneity of adenosine recognition sites on isolated myenteric varicosities and it was suggested that these sites may represent a heterogeneous population of functional adenosine receptors located on these nerve entities. Parallel functional evidence in support of heterogeneity of adenosine receptors was lacking. The ability to characterize the interaction of antagonists with the receptor present in the isolated intact guinea pig ileum and thus to obtain the affinities of the antagonists, permited possible receptor heterogeneity to be revealed in a functional system (Kenakin,1984).

Such studies, attempting to further characterize the receptor(s) at this site initially involved the use of the adenosine receptor antagonist theophylline (Daly,1985) in Schild analysis. Because competitive antagonism by methylxanthines is a characteristic of both A_1 and A_2 receptor subtypes (Daly,1985), it was of interest to



establish the affinity and nature of the interaction of theophyline with the adenosine receptor(s) at which A_1 and A_2 adenosine receptor analogs have equal potency. The results obtained in functional studies using the stimulated ileum (0.2Hz,1msec) clearly demonstrated the competitive nature of this antagonism. The linearity of the Schild plots and their unit slopes are indicative of a competitive interaction.

The affinity of theophylline for the receptor site(s) in the presence of NECA (as measured by the pA_2) was closely comparable to those obtained in the presence of the A_1-selective adenosine receptor agonists R-PIA, CHA, CPA and S-PIA (in the present studies) as well as adenosine (Okwuasaba and Cook,1980). A similar pA_2 was obtained for theophylline against R-PIA by Vizi et al.(1983). These data strongly suggest that both the 5'-N-carboxamide analog of adenosine, NECA, and the N^6-substituted adenosine analogs CPA, CHA, R-PIA and S-PIA interact at the same receptor. The parallel findings from binding studies of similar IC_{50} values for displacement of the specific binding of [^{3}H]-R-PIA and [^{3}H]-NECA by theophylline further support this contention and, in addition, clearly illustrate the utility of the PV preparation in characterizing functional adenosine receptors present on myenteric nerve terminals. It is possible that one or more putative adenosine receptor subtypes with hybrid characteristics are present on myenteric nerve endings of the ileum. However, a distinction could not be made because of the lack of A_1/A_2 receptor selectivity of theophylline.

It was therefore necessary to use a more selective antagonist in any further attempts to identify possible receptor subtypes. The xanthine analog DPSPX was selected for use in functional studies because of its apparent ability to distinguish different nucleoside binding sites on PV which may correspond to distinct adenosine receptors. Its reported selectivity (Daly,1985) and suitable solubility characteristics were also reasons for using it. At the time these studies were carried out, no other more selective antagonists were readily available.

The antagonist DPSPX yielded essentially comparable results for the A_1 agonists. The linearity of the Schild plots and their unit slopes were indicative of a competitive interaction. The similarity in the pA_2 values obtained for the A_1 agonists MCPA, CPA and CHA indicated the presence of a unitary adenosine receptor on myenteric nerve terminals. The higher affinity of DPSPX (pA_2 value 10 fold greater than that obtained with theophylline) at the peripheral adenosine receptor in comparison to that for theophylline, was consistent with the higher potency of DPSPX at biologically relevant adenosine receptors.

The Schild data for NECA was better described by a curvilinear isobole although its apparent pA_2 for DPSPX was similar to those obtained for the A_1 agonists. At an antagonist concentration of 5 uM, confidence intervals about the data points indicated a

significant ($p<.05$) difference between the dose-ratios obtained for the A_1 agonists and NECA. Deviations of the Schild isobole of this type are consistent with those derived on theoretical grounds for a heterogeneous receptor population having unequal receptor concentrations (Lemoine & Kaumann, 1984). It is possible that prejunctional neurotransmission in the ileum can be modulated by nucleosides via both an A_3 adenosine receptor as well as an A_2 adenosine receptor.

Curvilinearity of the Schild plot for NECA is also consistent with functional evidence presented by Gustafsson et al (1985) supporting the existence of postjunctional A_2 adenosine receptors. The characteristics of the direct smooth muscle inhibitory effects of nucleoside analogs, as described by these authors, support the presence of postjunctional A_2 adenosine receptors. Based on this report (Gustafsson et al,1985),the findings presented here suggest that NECA at low concentrations (10^{-9}-10^{-7}M) may interact with prejunctional adenosine receptors located on nerve terminals, whereas at higher concentrations which are required for effect when high concentrations of DPSPX are in contact with the receptor(s), NECA also interacts with postjunctional A_2 adenosine receptors. Simultaneous interaction of NECA at both an 'atypical' adenosine receptor (or an A_3 receptor) and a postjunctional A_2 adenosine receptor which possess distinct affinities for nucleosides, would be expected to result in a curvilinear Schild plot. The presence of a prejunctional A_2 adenosine receptor on myenteric nerve endings

should also be contemplated.

While curvilinearity of the Schild plot for NECA may support heterogeneity of adenosine receptors at this locus, additional influences on the form of the isobole must be considered . Another factor which may be contributing to this observation is the existence of non-equilibrium conditions, such as a veiled uptake process for this agonist (Kenakin, 1982). While this may have contributed to the observed finding, the possibility is unlikely since determination of the dose-effect curve for NECA in the presence of the uptake inhibitor NBMPR, showed no shift of the curve (Christofi and Cook, 1985). Even though heterogeneity of adenosine uptake sites has been demonstrated in brain membranes by labeled dipyridamole binding (Marangos et al,1985), interaction of NECA with such uptake sites is uncertain in the absence of any evidence supporting the presence of such uptake sites at the peripheral locus. All dose-effect data was obtained using a non-cumulative addition technique in which sufficient time was allowed for the maximum response to develop, ensuring as close to equilibrium conditions as possible. In conjunction with the binding data reported here which demonstrates the existence of a heterogeneous population of adenosine recognition sites for NECA on PV, such a curvilinearity suggests that the existence of more than one receptor site must be contemplated.

Characterization of adenosine receptors involved in the modulation of tachykinin release from myenteric nerves.

Adenosine inhibits the release of Ach from myenteric nerves (Vizi & Knoll,1976; Sawynok & Jhamandas,1976; Hayashi et al,1978) by interacting with prejunctional adenosine receptors (Gustafsson et al,1978; Christofi & Cook,1987. Although such release is now well characterized, the effect of nucleosides on the release of other excitatory neuromediators in the enteric nervous system is not known. Adenosine-mediated regulation of tachykinin release from myenteric nerves was investigated using the atropinized longitutinal muscle-myenteric plexus strip (LMMP). The present studies provide novel evidence that nucleosides inhibit the release of tachykinins from the myenteric nerves of the guinea pig small intestine.

The LMMP preparation was used because it is devoid of the submucosal plexus and enterochromaffin cells, but it contains the myenteric plexus of the guinea pig ileum. The vast majority of the TK in the LMMP layer is contained in neurons of the myenteric plexus (Costa et al,1980; Costa et al,1981) and may be released upon electrical stimulation or stimulation with the neuropeptide cholecystokinin octapeptide (CCK8).

The contractile responses to 5nM CCK8 or 20 Hz electrical field stimulation used in these studies, were mediated by tachykinin release from myenteric nerves since desensitization with SP or the presence of the tachykinin antagonist [d-Pro^{4},d-Trp^{7,9,10}] SP (4-11)

in the bathing medium almost abolished these responses. Since the SP-A does not selectively block SP, a-NK or b-NK- mediated contractions of the LMMP, it is not known which TK(s) is/are released upon stimulation. The loss of the response to CCK8 or ES was not due to a nonspecific depressant action of high concentrations of the SP-A on the smooth muscle, because the ilea showed no reduction in the contractile response to the nonspecific stimulus KCl, but responses to equieffective concentrations of CCK8 and SP were abolished. Further, the concentration of the SP-A used (40uM) was relatively low (4 times higher than its pA_2 obtained on the guinea pig ileum against SP; (Regoli et al,1984) to have caused nonspecific inhibition of contraction.

The specificity of the SP antagonist for blocking the interaction of TK's with their receptors was also illustrated by its inability to block contractile responses to KCl, while significantly blocking responses to histamine which has been shown to release SP via an H_2 histamine receptor interaction (Barker and Ebersole,1982). Therefore, the observation that responses to ES were only partially inhibited by the SP-A indicates that a portion of the response is not mediated by TK's. That portion may involve release of other excitatory mediators from enteric nerves which may include serotonin and the products of the arachidonic acid cascade (Gustaffson,1980; Barker and Ebersole,1982).

The contractions to either 20Hz electrical stimulation or CCK8 were were nerve-mediated, since they were prevented by tetrodotoxin. Tachykinin release from myenteric nerves probably occurs by action potential conduction via Na^+ channels in a more excitable part of myenteric neurons (North,1982). At what point in the complex network of nerve fibers, CCK8 acts is not descernable from these studies. Similarly it is not possible to deduce from which part of the TK-nerves, TK's are released from, but since SP nerve fibers are varicose like other autonomic nerve fibers (Costa et al,1980), it may be suggested by analogy that the TK (s) is/are released en passage from the varicosities during conduction of a nerve impulse (Burnstock,1981).

The rate of electrically evoked release of SP-LI from LMMP is dependent on the frequency of stimulation (Baron et al,1983). The apparent abrupt transition and plateaus in the frequency dependence profile of SP release observed by Baron and co-workers (1983), suggested that its release from the myenteric plexus is multitonic. The frequency of stimulation (20Hz) used in the present studies is well within the range of frequencies which have been shown to maximally evoke release of SP from LMMP (Baron et al,1983). Since the release of a-neurokinin and b-neurokinin from enteric nerves has not yet been confirmed, the assumption was made that 20Hz is also appropriate for releasing these tachykinins.

The action of cholecystokinin octapeptide on the atropinized guinea pig ileum longitutinal muscle is mediated by the release of SP from myenteric nerve endings (Hutchison & Dockray,1981). Whether CCK-8 also releases a-neurokinin and b-neurokinin was not addressed in their study, since no specific antisera for a-NK and b-NK were available to directly answer this question. Equieffective responses to CCK8 (5nM) and ES were used in the present studies, to allow a valid comparison between effects of nucleosides on receptor mediated-(ie.CCK8) and electrically induced- contractions (ie.ES, 20Hz) of the LMMP.

Several selective A_1 and A_2 adenosine receptor agonists inhibited the contractile responses of the atropinized LMMP in a dose - dependent fashion. This data strongly suggested that nucleosides may regulate the release of TK's from myenteric nerve endings. Nucleoside inhibition of the CCK8-mediated release of tachykinins occured with a potency order of CPA > NECA > 2PAA, suggesting that the adenosine receptor mediating this response is similar to that involved in modulation of cholinergic transmission.

In contrast, the rank order of potencies obtained for electrically stimulated responses was CPA = NECA > 2PAA. Although not conclusive, the lack of difference in potencies between CPA and NECA may suggest that a distinctly different adenosine receptor modulates the release of tachykinins in comparison to that which modulates release of Ach, from myenteric nerves. This possibility is

consistent with the 780 fold selectivity of CPA for A_1 adenosine recognition sites in comparison to the A_2 site, revealed in ligand binding studies (Daly,1985). The potency difference between A_1 and A_2 adenosine receptor agonists would depend on the ratio of these and other, as yet unidentified, receptor subtypes in the enteric nervous system which mediate inhibition of transmitter release. It is reasonable to assume that electrical stimulation may be recruiting a population of myenteric neurons which releases tachykinins but is not subject to modulation by CCK-8. Thus, heterogeneity of adenosine receptors which are located on two distinct populations of myenteric tachykinergic nerve endings, may adequately explain the present findings.

In contrast to the CCK8-mediated contractions, the field stimulated responses were only partially inhibited by nucleoside analogs. These findings are not surprising, considering that field stimulation, besides releasing tachykinins, causes release of a variety of other excitatory as well as inhibitory substances which may contribute to the net contractile responses observed. These data are consistent with the findings of a partial blockade by the SP-A of the electrically-stimulated responses and strongly suggest that adenosine selectively inhibits the release of some but not all chemical messengers released at 20Hz stimulation. The nature of these other mediators is unknown. It is probable that some are neuropeptides, and several different neuropeptides which may be released at this frequency of stimulation have been identified in

the enteric nervous system (Furness & Costa,1981; McDonald et al,1988). Release of met-enkephalin (Glass et al,1984) and Ach (Paton,1957,1963; Cowie et al,1966) from myenteric neurons is inversely related to the frequency of stimulation over the range 0.1 - 80 Hz. These release profiles differ from those described for norepinephrine (Alberts and Stjarne,1982) and substance P (ie. 5 - 40Hz) (Baron et al,1983). Clearly then, different endogenous mediators may have varying contributions to the net contractile effect observed at a frequency of 20 Hz stimulation.

Substance P is the only tachykinin shown to be released from myenteric nerves by depolarizing stimuli (Holzer,1984; Baron et al,1983). Such release of a-and b-neurokinin from LMMP still remains to be demonstrated. Therefore, the possibility exists that nucleosides may modulate the release of one or more of the tachykinins from myenteric nerve endings. Concentrated PV suspensions were shown to contain substantial quantities of both a-neurokinin and SP-LI's. In contrast, only a relatively small amount of b-neurokinin was identified in PV with a SP antiserum having low cross-reactivity for b-neurokinin. In more dilute lysates of crude varicosity suspensions (P_2) there was no detectable b-neurokinin observed using an a-neurokinin antiserum which displayed approximately 50% cross-reactivity with b-NK and virtually no cross-reactivity with SP. Therefore, it is concluded that nucleosides regulate only the release of susbstance P and / or a-neurokinin from myenteric nerves of the guinea pig ileum, since b-NK is not likely

to contribute to the contractions produced by either ES or CCK8. The isolated nerve ending preparation may prove to be a suitable model system for identifying whether the release of both a-neurokinin and substance P is neuroregulated by nucleosides.

The affinity of selective A1 adenosine receptor antagonists for the putative adenosine receptor(s) present on enteric nerve endings were obtained from Schild analysis (pA_2 from x- intercept of isobole). The finding of linear isoboles with unit slopes was indicative of a competitive interaction at an adenosine receptor site which modulates release of TK's. The higher affinity displayed by CPDPX over DPSPX for the adenosine receptor(s) which mediate inhibition of CCK8-induced release of tachykinins is consistent with its higher affinity for A_1 adenosine receptors (Lee & Reddington,1986). Further, the affinity of DPSPX for the receptor site(s) in the presence of NECA was closely comparable to that obtained for the A_1 selective adenosine receptor agonist CPA, suggesting that these agonists interact at the same receptor site. Considering that the selectivity of DPSPX for binding to A_1 receptors is only 3.4 fold in comparison to that for A_2 receptors, it may not have been sufficient to clearly discriminate between the affinities of these receptor subtypes. It is concluded that CPA may be a better discriminator for identifying A_1/A_2 subtypes of adenosine receptors at the myenteric locus.

The affinity of these antagonists (pA_2) for the adenosine receptor(s) which regulates the CCK-8-mediated release of tachykinins is the same as that for the receptor(s) which regulate release of Ach (Christofi & Cook,1987). It is concluded that endogenous adenosine may modulate the release of both Ach and TK's by interacting with similar adenosine receptor subtype(s). Further, a different adenosine receptor may regulate the release of tachykinins from a CCK-insensitive population of tachykinergic nerve endings.

Adenosine receptor(s) may be located on the same population of nerve endings containing both TK's and Ach. However evidence for co-localization of TK's and Ach in the same nerve endings is lacking. Although SP containing nerve endings (derived from LMMP) have been shown to co-distribute with nerve endings utilizing [^{3}H]-Ach on sucrose density gradients (Bucsics et al,1986),this does not exclude the possibility that separate populations of TK and Ach - containing nerve endings of similar density co-distribute on such gradients. Agoston et al (1985) showed that SP-containing vesicles from myenteric varicosities of guinea pig LMMP could be separated from vesicles containing Ach by density gradient centrifugation indicating that Ach and SP are stored in different neuronal pools. As it is not clear if SP and alpha-NK are co-localized in the same neuronal pools (Deacon et al,1987) it is conceivable that adenosine regulates the release of Ach, SP and alpha-NK from separate neuronal pools, by interacting with the same adenosine receptor(s).

Nucleosides may directly or indirectly modulate release of TK's from enteric nerve endings. Indirect inhibition may involve the cholinergic input to TK-containing nerve endings described by Franco et al(1979) and Holzer (1984). That is, nucleosides interact with adenosine receptors present on varicosities to block the release of Ach which in turn interacts with nicotinic receptors on TK-ergic varicosities to release less TK's. This is unlikely to be the case for two reasons: First, addition of hexamethonium (0.4mM) to the bath medium had no effect on the electrically (20Hz) induced release of SPLI (Holzer,1984). Secondly, the high micromolar concentrations of Ach required to release SPLI is unlikely to be released upon ES of the LMMP. Thus, it is concluded that nucleoside inhibition of the release of both Ach and tachykinins occurs through interaction with similar adenosine receptors which are likely to be located on separate cholinergic and tachykinergic nerve endings.

In contrast to the present report of nucleoside inhibition of depolarization-induced release of TK's from myenteric varicosities, studies using rat spinal cord slices (Vasko et al,1988) failed to demonstrate inhibition of depolarization-induced release of SP by nucleosides . It is possible that modulation of SP release from neurons in the spinal cord does not involve an A_3 adenosine receptor (Ribiero and Sebastiao,1986) which is believed to be linked to a voltage-sensitive calcium channel. Alternatively, the data may suggest that adenosine receptors present on myenteric varicosities

which inhibit the release TK's are not generally present on all neurons which release TK's. Selective localization of adenosine receptors would be consistent with a physiological role for adenosine in neuroregulation of enteric tachykinergic transmission.

Preliminary studies on the use of isolated myenteric varicosities as a functional system to study neuroregulation of peptide release by nucleosides.

Radioimmunochemical and immunohistochemical studies indicated that many peptides are localized in neurons of the myenteric plexus. The functional implication of such a localization, is that these peptides may act as transmitters or neuromodulators in the enteric nervous system. The role of nucleosides in the neuroregulation of the release of these peptides from enteric nerves is poorly understood. It was thus of considerable interest to develop a suitable in vitro model system for the investigation of nucleoside regulation of endogenous peptide release from myenteric nerve endings.

The utility and suitability of the isolated myenteric nerve ending preparation in characterizing biologically relevant adenosine receptor(s) has already been established from ligand binding studies. This work resulted in the characterization of nucleoside receptor(s) at PV which are involved in regulation of neurogenic responses to both tachykinins and Ach from enteric nerves.

Previous reports (Holzer,1983; Furness & Costa,1981; Baron,1984) using the stimulated ileum preparation showed that SPLI is released from enteric nerve endings. However, these studies did not specify the cross-reactivity of antisera with a-neurokinin and b-neurokinin. Therefore, these authors may have been measuring release of these other mammalian tachykinins from enteric nerves in addition to SPLI but evidence for such release is still lacking. With the recent development of specific antisera for a-neurokinin it was now possible and necessary to re-evaluate the nature of the 'SPLI' believed to be released from enteric nerve endings. Demonstration of depolarization-induced release of these other mammalian tachykinins would strongly support their role as neurotransmitters and/or neuromodulator in the enteric nervous system. Functional studies presented here using the atropinized LMMP preparation, were unable to identify specifically which tachykinin(s) are under neuroregulation by nucleosides. Clearly then, studies were required to elucidate, both the identity of the tachykinin(s) released from isolated myenteric nerve endings, as well a their possible neuroregulation by nucleosides. Answers to such fundamental questions would support a physiological role for nucleosides in neuroregulation of peptidergic nerve function in the enteric nervous system.

As the isolated myenteric nerve ending preparation had not been used in the past for measuring endogenous release of neuropeptides, it was necessary to first establish both the feasibility and

suitability of this model system for such studies. The approaches used to characterize this in vitro model system included:

i) Identification and quantitation of the chromatographic forms of the tachykinins, as well as other peptides, present in PV.

ii) Correlation of neuronal content of peptides in various fractions involved in the isolation of partially purified nerve endings, with the Occluded LDH activity (a measure of cytoplasm enclosed by a membrane) which would establish which fraction(s) were appropriate for use in release studies.

iii) Characterization of the basal release of neuropeptides which could provide useful information on the possible interactions between synaptosomes which mav occur in a heterogeneous suspension of myenteric nerve endings.

iv) Evidence for the presence of functional Ca^{++} channels on enteric nerve endings which would be crucial in order to test Rebiero and Sebastiao's (1986) hypothesis that the putative A_3 adenosine receptor located on these nerve terminals, acts by blocking voltage sensitive Ca^{++} channels.

iv) Release of tachykinins and other neuropeptides from enteric nerve endings by the depolarizing stimulus K^+ which would further support the presence of voltage sensitive Ca^{++} channels at this locus. It would also suggest the the synaptosomes have an appropriate membrane potential for measuring depolarization induced release. Direct measurement of the membrane potential of the synaptosomes by the TPP^+-cation method would reveal the depolarization state of the synaptosomes for release studies.

v) Determination of K^+ evoked-release of tachykinins and enkephalins in the presence of antagonists of known endogenous inhibitory mediators (e.g.nucleosides and enkephalins) which could provide indirect evidence for their ability to tonically inhibit the release of neuropeptides from isolated myenteric nerve endings.

Characterization of content and chromatographic forms of neuropeptides in PV.

Studies were carried out in order to quantitate and characterize the chromatographic forms of several neuropeptides in a preparation of isolated myenteric varicosities isolated from guinea pig ileal LMMP strips. The objectives were to validate the RIA systems employed and to assess the suitability of the PV as a model system in studies on release and modulation of release of endogenous mediators.

RIA dose-inhibition relations of several neuropeptide-like immunoreactivities in PV produced dose-inhibition curves close to parallel or parallel with those produced by their respective synthetic peptides. These data suggested that the immunoreactive entities measured by the RIA systems represented peptides structurally very similar to their respective synthetic peptides and therefore served to validate the RIA systems employed.

The immunoreactive entities measured in this study are present in enteric nerve varicosities derived from the guinea pig ileal myenteric plexus. The inability of sensitive RIA systems to detect

either gastrin- or PYY-LI is consistent with no contamination of the PV preparation by endocrine elements. These two peptides are predominantly localized to mucosal endocrine-type cells.

In accordance with previous immunohistochemical studies (Schultzberg et al.,1980), SPLI was found in the highest concentration in the PV preparation in comparison to other immunoreactive entities measured. A dense innervation of all layers of the gut with VIPLI-containing nerves (Schultzberg et al.,1980) has also been reported and, considering the low cross-reactivity of the VIP antiserum used in this study with guinea pig VIP (28 X less), it is apparent that guinea pig enteric varicosities must also be a rich source of this neuropeptide. The relatively low concentration of GALLI in the PV is not unexpected, as nerve-cell bodies containing galanin immunoreactivity occur mostly in the submucous plexus of the small intestine (Melander et al.,1985). Both Met- and Leu-enkephalin were present in the PV preparation with Met-enkephalin being the predominent peptide in agreement with previous tissue extraction studies (Hughes et al.,1977).

The differing amounts of immunoreactive entities in the synaptosomal preparations found in this study probably do not accurately reflect relative concentrations in vivo. During isolation of PV, differing rates of depletion from storage sites, which may occur among the various peptides, would result in artifactually different relative levels. However, the SPLI content in the PV preparation was close to

the value reported by Buscics (1986) in a similar guinea pig synaptosomal preparation, indicating that PV yields a reproducible content of SPLI. Differential depletion of these neuropeptides from intraneuronal storage pools may exhaust the releasable pools of neuropeptides such that no detectable release can be measured with appropriate releasing agents.

Reverse-phase chromatography of the PV preparations revealed a number of SPLI peaks. Using 'steep' gradient elution conditions, major SPLI peaks occurred close to the retention times of synthetic substance P and its oxidized form suggesting that guinea pig substance P has, as suggested by Murphy et al.(1982), close identity to, or is structurally identical with substance P from other species. Since the SPLI peak consistently eluted slightly later than synthetic SP under high resolution HPLC conditions, a minor structural difference could not be completely excluded. A small but significant peak of SPLI occurred at an identical retention time to that of a-neurokinin, suggesting the presence of this newly identified mammalian tachykinin in guinea pig myenteric plexus and that guinea pig alpha-neurokinin is structurally similar to its porcine counterpart. Further, given the low degree of cross-reactivity of the SP antiserum employed with a-neurokinin, the amounts of the a-neurokinin-like entity present in the guinea pig PV preparation must be considerable. This would be in agreement with a recent study showing that guinea pig intestinal tissue extracts contain more immunoreactive a-neurokinin than SP (Hua,1985). More

recent studies using specific a-NK and SP antisera to detect these peptides in guinea pig synaptosomal lysates (P_2) indicated that similar amounts of these tachykinins exist in guinea pig P_2. As such, a-neurokinin, may also be a peptide with a physiological role in the enteric nervous system of the small intestine.

Due to the low cross-reactivity of the antiserum used in this study with b-neurokinin, it cannot be concluded that b-neurokinin is absent from these preparations. In more recent studies, using an a-neurokinin antiserum with approximately 50% cross-reactivity with b-neurokinin and virtually no cross-reactivity with SP, no b-NK immunoreactive peak was detected in P_2 lysates. If b-NK is present, it is present in a very low concentration relative to the other tachykinins in the enteric nervous system or it is absent from enteric varicosities and plays no role in enteric neurotransmission. These observations are consistent with previous findings that extracts of guinea pig small intestinal tissue (Deacon et al.,1987), extracts of capsaicin-sensitive neurons in guinea pig lung and ureter (Hua et al.,1985) and extracts of carcinoid tumors of the mid-gut (Theodorsson-Northeim et al.,1985; Conlon et al.,1986) contain no b-neurokinin. The successful production of a specific b-NK antiserum with low cross-reactivity for the other mammalian TK's will allow this question to be answered. Greater depletion of b-neurokinin from synaptosomes during isolation as compared to the other tachykinins could also explain the inability to detect this neuropeptide. This may suggest that b-neurokinin is exposed to

distinct enzyme systems intracellularly, implying distinct compartmentalization or a different sensitivity to those enzymes in comparison to the other tachykinins. Alternatively, if extremely low concentrations of b-neurokinin exist in comparison to the other tachykinins, then it is conceivable that distinct receptors, a distinct sub-population of varicosities containing only b-neurokinin and/or a distinct function may be ascribed to b-neurokinin in the enteric nervous system.

Using specific antisera for a-NK and SP, P_2 sample blanks obtained for a-NK were relatively very high in comparison to those obtained for SP at lower dilutions of the samples. This surprising difference is difficult to interpret at this point. The a-neurokinin tracer appeared to be binding to distinct a-neurokinin recognition sites. Attepts to remove the subcellular component in P_2 suspensions which binds a-neurokinin by ultracentrifugation at 105,900 X g of the S_3 supernatants were unsuccessful, indicating that a-neurokinin is binding to a minor constituent of the S_3 supernatants, smaller than microsomes and smooth muscle membranes. Clearly, an important difference between the binding characteristics of a-neurokinin and Substance P-like immunoreactivities exist. Conceivably, a-NK binding sites represent fragments of distinct a-neurokinin receptors present on enteric nerve endings.

A major peak of SPLI was present which had a considerably greater reverse-phase retention time than any of the known TKs or their

oxidized products, and hence was unlikely to have been an oxidized form of a known TK. It seems unlikely that it was either the recently described N-terminally extended form of a-neurokinin, called neuropeptide K (Tatemoto et al., 1985) or the eledoisin-like immunoreactive entities which have recently been described in guinea pig tissue extracts (Hua et al,1985) since, under similar HPLC conditions to those employed in this study, these entities eluted before b-neurokinin (Hua et al,1985) in contrast to the unidentified SPLI entity which eluted later. Similarly, the unidentified SPLI peak elutes later than any of the C-terminal framgents of SP. This SPLI could represent an N-terminally extended form of SP which, to date, has not been chemically characterized. Alternatively, although perhaps less probable but potentially of more interest, this SPLI peak may represent a previously unidentified mammalian tachykinin. Isolation and chemical characterization of this SPLI peak is necessary and could prove to have biological importance.

On reverse-phase HPLC, synaptosomal Leu- and Met-enkephalin-like immunoreactivities eluted at identical retention times to those of synthetic Met- and Leu- enkephalin, suggesting close structural identity of guinea pig enkephalins to those of other mammalian species. The small unidentified MELI peak may have represented one of the described C-terminally extended enkephalin forms.

As recently reported (Murphy,1984), a single VIPLI entity eluted at the retention time of porcine VIP under 'steep' gradient elution

HPLC condition but using 'shallow' gradient elution conditions, VIPLI eluted at the retention time of guinea pig VIP instead, which is clearly different from that of porcine VIP. The structure of guinea pig VIP differs by 4 amino acid residues (Du et al.,1985) from the previously reported structure of VIP which is identical in pig, cow, human, and rat (Carlquist et al.,1979; Carlquist et al.,1982; Itoh et al.,1983; Dimaline et al.,1984). These findings underline the importance of comparing retention times of peptides under multiple and high resolution HPLC conditions. GALLI elutes at a considerably different retention time from that of porcine galanin suggesting structural differences between the two peptides.

GRPLI occured in two peaks of immunoreactivity on reverse-phase HPLC. One peak eluted close to porcine GRPLI and the second close to GRP-(18-27) and the principal oxidized form of GRP-(1-27). The C-terminal portion of porcine, canine, human and avian GRP molecules is mainly conserved but multiple conservative amino acid interchanges are present in the N-terminal region (McDonald et al.,1979; McDonald et al.,1980; Spindel et al.,1984; Reeve et al.,1983). The HPLC data obtained in this study, however, suggest a close structural identity of guinea pig GRP with porcine GRP. Previous studies have demonstrated the presence of substantial quantities of the decapeptide, GRP-(18-27), in freshly extracted canine small intestinal tissue (Reeve et al.,1983). At least part of the GRPLI in the early eluting peak could be due to GRP-(18-27).

The presence of oxidized forms of some of these neuropeptides enhanced identification in this study, but performance of future functional experiments will have to take this factor into account. For example, the conduct of organ bath experiments in which the LMMP is bathed in gassed Krebs' solution, may result in oxidation of exogenously added or endogenously released neuropeptides during ES, which may inactivate or alter the bioactivity of the peptide. Suspension of P_2 or PV synaptosomal preparations in gassed Krebs may similarly alter the bioactivity of exogenous peptides or endogenously released peptide. The similarity of several of the neuropeptides present in the PV preparation, as determined by reverse-phase HPLC elution characteristics, to synthetic counterparts, and the parallel or close-to-parallel dose-inhibition curves obtained, indicated that these entities could be quantified by the RIA systems employed.

The identification of substantial quantities of SPLI, a-NKLI, MELI, LELI, GRPLI, GALLI and VIPLI in isolated myenteric nerve endings suggested that this in vitro preparation may prove useful in release studies involving neuropeptides. Demonstration of the presence of a-neurokinin and possibly of an unidentified mammalian tachykinin in the guinea pig myenteric plexus suggest that a re-evaluation of the nature and function of previously documented 'SPLI' pathways present in mammalian gut is essential. The majority of the functional studies focus on release and modulation of release of SPLI without any mention of the other tachykinins. It is very probably that a

significant portion of the responses measured are mediated by a-neurokinin.

Relative distribution of peptide-like immunoreactivity and occluded LDH activity in various fractions obtained by differential centrifugation of homogenates of guinea pig ileal LMMP.

The decrement in yield obtained with each successive step involved in the isolation of myenteric varicosities represents a measure of purification in the procedure. The Fb fraction represents a 5.6 x 10^3-fold purification of the starting material (LMMP). The gradient fractions collected from bands Fa, Fb and Fc occur at sucrose densities corresponding to mainly synaptic vesicles (Agoston et al,1985; Bucsics et al,1986) varicosities and mitochondria respectively (White & Leslie,1982;Yau & Verdun,1983).

Since Occluded LDH is a cytoplasmic enzyme marker, it was used as measure of the relative enrichement of subcellular fractions with intact varicosities. These studies showed that the highest concentration of intact myenteric varicosities / 1 guinea pig ileum was present in the P_2 followed in decreasing order by Fb, Fc and Fa. This order is consistent with the relative distribution of synaptosomes across sucrose gradients discussed earlier. Furthermore, these findings suggest that some intact varicosities may exist in the fraction of lowest density (Fa,.648M).

GRP, LE and VIP-LI's measured, form a broad band over a relatively wide sucrose density range, since they were detected in all three gradient fractions. Therefore the possibility of separating LMMP varicosities containing these neuropeptides on the basis of their sedimentation properties on sucrose gradients is unlikely.
In this respect, Bucsics & co-workers (1986) showed that SP-containing myenteric nerve endings co-distribute with nerve endings utilizing NA or Ach on sucrose density gradients (see also Jonakait et al,1979). The apparent inability to separate subpopulations of myenteric varicosities on the basis of density is of functional importance in that mediators released from one type may modulate the function of another.

The highest concentrations of GRP, VIP, ME and LE-like immunoreactivities were present in the P_2 (fmol/guinea pig) in agreement with its occluded LDH activity, and with the exception of MELI, the recovery of these neuropeptides from the gradient fractions was about 50%. Therefore, considerable neuropeptide loss occured during the steps beyond the P_2. Depletion of neuropeptides from the varicosities, damage during isolation together with their wide distribution on continuous gradients may contribute to such losses. In contrast, recovery of MELI on continuous sucrose gradients was approximately 10%, indicating that its neuronal loss during isolation is the greatest. The possibility exists that a separate subpopulation of varicosities containing ME distributes differently on continuous sucrose gradients as a result of its

distinct density characteristics. However, it is more likely that a relatively greater depletion rate of ME from varicosities explains this difference. This possibility is supported by the high basal release of ME in comparison to that observed with the other peptides.

Since more neuronal peptide exists at the P_2 stage (ie.especially true for MELI), this fraction may prove to be more suitable for conducting neuropeptide release experiments from varicosities.

Relative neuropeptide contents (fmol/animal) do not correspond to relative occluded LDH activities across the gradient fractions. One possibility to explain this discrepancy is that immunoreactivities measured, particularly in the Fa fraction, reflect some peptide present in intact varicosities but mostly peptide contained in free synaptic vesicles. If GRP, ME, LE and VIP-like immunoreactivities of the Fa fraction are expressed as fmol/mg Fa protein, enrichment in immunoreactivities of this fraction over the P_2 occur in the order of 4.4 -, 1.14 -, 4.77 - and a 3.04 - fold respectively. This enrichment probably represents concentration of synaptic vesicles in the F_a fraction. It is important to note that no increase in MELI content occured in this fraction. This is in keeping with the possibility that a separate, intact population of met-enkephalinergic nerve endings exists containing MELI but not the other immunoreactive entities. Alternatively, met-enkephalin may be distributed in a separate population of denser synaptic vesicles

which do not co-distribute with vesicles containing GRP, LE or VIP.

Use of substance P as the peptide to investigate the conditions suitable for studying release of endogenous peptides from isolated nerve endings.

It has been suggested that the presence of EGTA in the isolation medium is important for allowing the nerve endings to respond subsequently to depolarization (Cazalis et al.,1987). Using atomic absorption spectrometry, the [Ca^{++}] concentration of a medium without added [Ca^{++}] and EGTA was determined to be approximately 30uM Ca^{++} (free + bound to membranes). As this might be a high enough concentration to inactivate some internal mechanisms or to cause release during homogenization, 50uM EGTA was included in the isolation medium. In support of this possibility, it was observed that the basal release of SPLI was significantly lower in preparations which were isolated in Krebs or Hepes buffer containing normal amounts of Ca^{++} (2.54mM) in comparison to control preparations isolated in 0.32M sucrose. The depletion of releasable pool(s) of SPLI and/or inactivation of the basal release process may have contributed to the observed findings.

The isolated myenteric nerve ending preparation is a rich source of degradative enzymes as indicated by its rapid degradation of exogenously added substance P. The protease inhibitor bacitracin alone was shown to be more effective than the mixture of leupeptin, chymostatin and bacitracin in protecting against degradation of

exogenous SP. Therefore, bacitracin alone was routinely included in the incubation mixture. The enzyme activity which is inhibited by bacitracin has not yet been characterized (Horsthemke et al,1984).

The enhanced basal release of SPLI from the P_2 synaptosomes observed in the presence of bacitracin demonstrates the ability of this protease inhibitor to retard degradation of endogenously released SPLI. The basal release of SPLI was dependent on the concentration of bacitracin in the incubation mixture. With resuspension of synaptosomes in Locke's solution, the basal levels of SPLI reached a plateau at 5mM concentration of bacitracin. In contrast, a linear increase in basal release of SPLI dependent on the concentration of bacitracin was observed when synaptosomes were resuspended in Krebs buffer (gassed with 95%O_2;5%CO_2). Hepes buffer was used in all subsequent experiments focussing on release of neuropeptides.

Considerable depletion of the neuronal content of SPLI occured during equilibration of the synaptosomes in Krebs at 37 C, which could be explained by the high ongoing spontaneous release of SPLI. About 50% of the tissue content was depleted within 1.0h of equilibration. Since depletion of neuronal stores of SPLI probably also depleted the releasable pool(s) of this immunoreactive material, it was of importance to assess the influence of the equilibration time on released peptide (see below).

Basal release of neuropeptides from P_2 synaptosomes derived from LMMP strips of the guinea pig ileum.

The basal release of several peptide-immunoreactivities from isolated myenteric nerve endings is not predictable on the basis of their neuronal contents. The rate of spontaneous release of the enkephalins (MELI and LELI) is the highest whether the amount is calculated as an absolute concentration or as a percentage of neuronal content. Among the enkephalins, MELI is released in the highest concentration. In contrast, the tachykinins which are present in the highest concentration in the varicosities, are spontaneously released in lower amounts than the enkephalins. Since release of one neuropeptide may affect the release of another from enteric nerve endings, such basal release may have a significant effect on the state (i.e.active vs inactive) of ion channels present in the synaptosomal membrane. Such possible tonic effects must be considered in the interpretation of findings from release studies with various drugs. Given that the enzymatic degradation of substance P and probably a-neurokinin are essentially completely inhibited in the presence of bacitracin (5mM) used in these studies, it is likely that significant differences between the relative tonic levels of basal release of tachykinins and enkephalins exist at this preparation.

An accurate determination of the time course of basal release of these peptides following resuspension of the synaptosomes is essential in order to study evokable release of peptides, as indicated by the time-dependent decrease in basal release of SPLI from P_2 synaptosomes. It is possible that a portion of the initial high basal release of SPLI (at 0 min. equilibration) represents damage to synaptosomes following resuspension, while the lower rate of release observed after 60 min. equilibration represents spontaneously evoked release of SPLI. The resuspension of synaptosomes is likely to cause non-specific disturbances to ion fluxes across the synaptosomal membrane (i.e. functional damage) which could in part explain the high initial basal release. It is also possible that, the basal release of SPLI diminishes as a result of a time-dependent decrease in release of an excitatory mediator or accumulation of an inhibitory mediator, in the incubation mixture. This is an intriguing possibility, since a portion of the basal release may represent functional release of SPLI from synaptosomes and not damage.

Evidence for functional basal release of several peptide-immunoreactivities was obtained in experiments with the calcium channel antagonist methoxyverapamil (D600, 100uM). D600 inhibited approximately 50% of the basal release of a-neurokinin, substance P and leu-enkephalin, strongly suggesting that such basal release is dependent on voltage-sensitive Ca^{++} channels. At least this portion of the basal release may represent a modulated release resulting

from the tonic effects of endogenous mediators (ie. neuropeptides etc.). The concentration of D600 used (100uM) was reported to inhibit both the evoked release of AVP from isolated neurosecretory nerve endings (Cazalis et al.,1987) and the $[Ca^{++}]_i$ into neurosecretosomes isolated from anterior pituitary (Brethes et al,1987). Non-specific effects of D600 could not explain its inhibitory action on basal release of neuropeptides, since such effects are believed to occur mainly at concentrations > 100uM. More specific Ca^{++} channel blockers are the dihydropyridines (Cazalis et al.,1987) which have been shown to block SP release from DRG neurons (Perney et al,1987), a finding consistent with a major participation of L-type channels.

The findings with D600 support the contention that the varicosities under basal conditions are in a state of excitation, given that D600 is blocking the influx of extracellular Ca^{++} through open voltage sensitive Ca^{++} channels on varicosities. As alluded to previously, endogenously released substances under basal conditions (possibly neuropeptides) may function by opening voltage-sensitive Ca^{++} channels accounting for the D600-sensitive component of basal release. As a consequence it would be more difficult to cause further K^+ depolarization-induced release of a-neurokinin and SP from these nerve endings. This may offer an explanation for the inability of K^+ depolarization to evoke release of the tachykinins (see below). Clearly though, the basal release of both a-neurokinin and SP are Ca^{++}-dependent processes, arguing against the possibility

that basal release is due to nonspecific damage of the varicosities. In experiments in which the P_2 synaptosomes were isolated in Ca^{++} - containing buffer instead of 0.32M sucrose and EGTA, the subsequent basal release, as well as neuronal content of a-neurokinin and substance P-like-immunoreactivities measured, were significantly lower. This finding is consistent with extracellular calcium - dependent depletion of the tachykinins by continuous release from the synaptosomes and provides indirect evidence supporting the release of SPLI from isolated myenteric nerve endings.

Ribeiro and Sebastiao (1986) suggested that inactivation of voltage-dependent calcium channels on myenteric nerve terminals may be the mechanism by which adenosine interacts with an A_3 adenosine receptor to inhibit transmitter release. Therefore, it is suggested that the calcium-dependent basal release of a-neurokinin and substance P demonstrated here may serve as suitable responses in functional studies of neuroregulation of tachykinin release from myenteric nerve terminals by nucleosides.

Observations made from studies involving the depolarizing stimulus K^+, suggest that the evoked release of the enkephalins (MELI and LELI) and the tachykinin a-neurokinin are dependent on the spontaneous release of these peptides. In all cases where release of these peptides was observed, a clear inverse relationship existed between their initial spontaneous release and their K^+-evoked release. The most prominent level of release was achieved in

preparations having the lowest spontaneous release. The lowest amount of release was evident at preparations having the highest basal release. Since the K^+-evoked release of these peptides appeared to be dependent on the magnitude of their spontaneous release, it is suggested that their basal release reflects the depolarization state of the synaptosomes. In preparations with higher basal release and hence with more positive membrane potentials, it is expected that it would be more difficult to cause further release with a depolarizing stimulus.

Approximately 25% of the basal release of each TK may be attributed to an inappropriate centrifugation speed used for terminating the release reaction. Centrifugation of the P_2 suspensions was carried out at 13,000 X G which is sufficient to bring down most of the synaptosomes. However, smaller, less dense synaptosomes and synaptic vesicles may be left behind in the supernatants and contribute to the basal release observed. The finding that equal amounts of SP and a-NK (25%) are recovered from the supernatants following a faster centrifugation (105,900 X G) may indicate that such subcellular components are similar in density. In light of the recent report (Deacon et al.,1987) that a-neurokinin and Substance P synaptic vesicles derived from small intestine, co-sediment in sucrose density gradients, it is possible that synaptic vesicles containing the two tachykinins represent the remaining 25% immunoreactivities in the supernatants. Alternatively, nonspecific binding of neuropeptide to membrane fragments may account for these

immunoreactive fractions. Clearly, 75% of the immunoreactivity of the tachykinins basally released is the maximum which may be inhibited by various exogenously added substances. This finding is of importance in the interpretation of data obtained from studies on neuroregulation of the basal release of tachykinins from isolated myenteric nerve endings.

Depolarization-induced release of neuropeptides from isolated myenteric nerve endings.

Initial experiments involving the depolarizing stimulus K^+, failed to demonstrate evoked release of tachykinins from P_2 varicosities. Thus, any further studies with depolarizing stimuli were carried out only in the presence of antagonist(s) of known endogenous inhibitory mediators as a means of testing the hypothesis that endogenously released mediators, under basal conditions, influence the depolarization state of the varicosities.

The depolarizing stimulus [K^+] caused a dose-dependent release of MELI only in synaptosomal suspensions which contained either naloxone or atropine. The demonstration of evoked release of this neuropeptide with the depolarizing stimulus K^+ is consistent with a neurotransmitter and/or neuromodulator role for ME in the enteric nervous system. This finding also confirms that the P_2 contains entities with an appropriate V_m and that they can undergo depolarization leading to an exocytotic release process.

Given that the P_2 represents a heterogeneous population of nerve endings it is important to note that the release of ME with elevation of K^+ during the 3 min. reaction represents a net release resulting from the influence of all excitatory and inhibitory mediators co-released with ME during depolarization. Different populations of nerve endings may have different states of depolarization as a result of the interactions between synaptosomes. Therefore, any early release of mediators which inhibit or stimulate ME release may contribute to the net ME release caused by depolarization.

The need for high concentrations of K^+ to evoke release may in part be also explained by depolarization of the synaptosomes by endogenously released substances under basal conditions. If the synaptosomes were already relatively depolarized under basal conditions, further depolarization with elevated $[K^+]$ would be more difficult. The high basal release of MELI from the synaptosomes supports this contention.

In the presence of the selective A_1 adenosine receptor antagonist DPSPX in the incubation mixture, elevated K^+ failed to evoke release of MELI, arguing against the involvement of endogenously released adenosine in neuroregulation of the release of MELI under the present assay conditions. Even though elevated $[K^+]$ evoked release of MELI in the presence of naloxone, such release was not evident when the incubation mixture contained the mixture of naloxone, DPSPX

and atropine. In contrast to MELI, depolarization-induced release of LELI was evident only in the presence of this mixture of antagonists, suggesting that complex interactions may occur between varicosities in this heterogeneous population of nerve endings and, in the absence of the appropriate antagonist(s), net release may not occur. Such studies with antagonists are difficult to interpret in the absence of detailed knowledge of the location and nature of opioid, adenosine and muscarininic receptors on enteric nerve endings.

It is concluded, that evoked release of MELI and LELI from myenteric nerve endings depends on the type and combination of antagonists present in the incubation mixture. The findings are consistent with the presence of distinct subpopulations of nerve endings containing LELI or MELI, the release of which, may be under differential regulation by endogenous mediators.

In contrast to ME release, detectable release of SPLI from the same synaptosomal suspensions was not apparent in the presence or absence of the receptor antagonists. According to the [Ca^{++}] hypothesis of neurotransmitter release (Hubbard,1970), depolarization of nerve terminals (ie. elevated K^{+}) triggers an influx of Ca^{++} through voltage-sensitive Ca^{++} channels and the consequent elevation of free [Ca^{++}] within the nerve terminals activates neurotransmitter release. The release of SP has been shown to be [Ca^{++}]-dependent in both central and peripheral nerve endings (Holzer,1984). Sustained

inactivation of these voltage sensitive [Ca^{++}] channels by hyperpolarization of the tachykinin-containing varicosities (Cook, 1988) with endogenous neuromediators would attenuate or prevent any possible release of TK's by [K^+]. However, the inhibition of basal release of substance P with the calcium channel blocker D600 supports the presence of open [Ca^{++}] channels on varicosities, implying that these entities are in a state of relative depolarization rather than hyperpolarization. Relative depolarization of these varicosities would make it more difficult to further depolarize them with [K^+] which, in turn, would cause further release of SPLI.

Since GRP and bombesin evoke substantial release of SPLI under the same conditions used in the present study, the lack of effect of elevated [K^+] is unlikely to represent a problem of rapid degradation, depletion of releasable stores of TK's, or an RIA sensitivity problem. Instead it may reflect differences in the depolarization or excitation state of varicosities containing substance P. This would imply that substance P is present in a subpopulation of varicosities, which are under distinct regulatory control from those containing the enkephalins. Supporting evidence comes from studies which showed that the LMMP of the guinea pig ileum contains a population of met-enkephalin containing enteric neurons, distinct from those for SP (Furness et al,1983). The presence of naloxone in the incubation mixture is probably sufficient (Glass et al,1986) to block the inhibitory effect of

[K^+]-evoked release of MELI on any concomittent release of SPLI, thereby excluding the possibility that endogenous enkephalins themselves are responsible for the apparent lack of effect of K^+ on SP release.

Preliminary experiments with enteric nerve endings have demonstrated that the sodium ionophore monensin dose-dependently evoked release of a-NK and SP in the same preparations in which the calcium ionophore A23187 (50uM) failed to release tachykinins. These observations are consistent with the involvement of extracellular Na^+ influx in the release process under the conditions used. This apparent lack of effect of both depolarization and A23187 strongly suggests that extracellular calcium influx cannot cause any further release of the tachykinins (in the absence of any antagonists) than that which occurs under basal conditions. Considering that bacitracin does not totally inhibit enzymatic degradation of SPLI and probably a-NKLI, the portion of their [Ca^{++}]-dependent basal release measured may be an underestimation.

Release of the tachykinins with monensin indicates that the their releasable pool(s) have not been totally depleted during the isolation of the P_2 synaptosomes. Monensin released more a-neurokinin than substance P like-immunoreactivity from enteric synaptosomes, suggesting that differences in the [Na^+]-dependent component of release may exist between the tachykinins.

Clear depolarization-induced release of a-NKLI from P_2 synaptosomes was evident in the presence of the A_1-selective adenosine receptor antagonist DPSPX, but not in its absence, from the incubation mixture. In the presence of antagonists for opioid and muscarininic receptors (e.g. naloxone and atropine, respectively) [K^+]-depolarization failed to produce detectable release of a-neurokinin. This requirement for DPSPX in the incubation mixture, suggests that endogenous adenosine may be involved in the neuroregulation of release of a-NKLI from enteric synaptosomes. Endogenous adenosine/ATP which may be released spontaneously from myenteric nerve endings could be attenuating the initial depolarization-induced release of a-NKLI. The sharp decline in evoked release of a-NKLI may be attributed to the co-release of adenosine and ATP with a-NKLI during K^+-depolarization (White & Leslie,1982). This possibility is consistent with Ribeiro and Sebastiao's hypothesis (1986) that adenosine inhibits transmitter release by interacting with an A_3 adenosine receptor to block voltage-sensitive calcium channels on nerve endings. Direct demonstration of nucleoside inhibition of a-NKLI release from P_2 synaptosomes remains to be demonstrated.

The maximum release of a-NKLI observed with K^+ is essentially the same in three separate experiments. This strongly suggests that a finite and reproducible pool of a-NKLI exists in varicosities which may be released upon depolarization. Assuming that this pool of a-NKLI is released by the influx of calcium through voltage dependent

calcium channels, then it represents a small component (7%) of the neuronal content of a-NKLI. Release of enkephalins from varicosities represents about 30% of their neuronal content. These findings support the views that either varicosities containing MELI possess a different depolarization state than those containing a-neurokinin, or different size releasable pools exist for these neuropeptides.

In the same experiments in which clear release of a-NKLI was obtained (ie. in the presence of DPSPX), no detectable release of SPLI was observed. This data seems to suggest that endogenous adenosine, which may be released either under basal or stimulated conditions, modulates release of a-neurokinin but not substance P. It is tempting to speculate that the tachykinergic contractions, which were shown to be inhibited by nucleosides, may have involved the release of predominantly a-NKLI and not SPLI. In previous studies on release of SPLI from LMMP (Furness & costa,1981; Holzer,1983; Baron,1983), the cross-reactivity of the SP antiserum with a-neurokinin was not reported since, at that time, little was known about a-NK. Resolution of this important question, requires direct demonstration of nucleoside inhibition of the depolarization-induced release of a-NKLI and not SPLI from the synaptosomes.

Such differences between depolarization-induced release of a-NKLI and SPLI from myenteric nerve endings may reflect more fundamental differences between the release processes for these tachykinins. These functional differences are also consistent with the existence

of distinct populations of myenteric nerve endings containing substance P or a-neurokinin. The demonstration of [K^+]-evoked release of a-NKLI from the synaptosomes is a novel finding supporting the neurotransmitter/neuromodulator role of this tachykinin in the myenteric nervous system.

The threshold concentration of [K^+] required to evoke release of a-NKLI from the synaptosomes was lower than that required to release MELI and LELI in the same experiments. The release of a-NKLI occurred at [K^+] concentrations of 6mM - 16mM, whereas release of the enkephalins occurred at relatively higher [K^+] concentrations in the incubation mixture (usually greater than 50 mM K^+ required). Such clear differences are consistent with the presence of distinct populations of myenteric varicosities containing a-neurokinin or enkephalins-like immunoreactivities. These distinct entities may be at different resting membrane potentials as a result of differential regulation of their release by endogenous mediators.

Functional studies using the LMMP strip showed that striking differences exist between the frequency-dependence profiles for the release of enkephalin-LI (Glass et al,1986) and the release of SPLI under identical experimental conditions (Baron et al,1983). In support of the present findings, these authors suggested that differences in the frequency-dependence profile for the release of peptides from the myenteric plexus could help identify the particular fiber types that transmit via each putative peptide transmitter. By analogy, differences in the [K^+] dependence profile

for the release of peptides from isolated myenteric nerve endings could help identify different functionally different sub-populations of nerve endings.

GalLI is also not released by [K^+] depolarization in the presence or absence of any of the aformentioned antagonists. Given the low neuronal content of GalLI in the synaptosomal preparation, it is possible that GalLI is released but is below the detection limit of the RIA. This would be especially true if rapid degradation of GalLI occurs. Depletion of the releasable pool of GalLI from the synaptosomes can not explain the lack of release, since bombesin evoked release of this peptide under identical isolation conditions. Although speculative at this point, lack of GalLI release due to [K^+] depolarization may imply that a [Ca^{++}] independent release mechanism is involved as has been demonstrated for VIP in the enteric nervous system (Belai et al,1987). Although release of established neurotransmitters (Kelly,1979) and neuropeptides like SP (Holzer, 1984) has been shown to be dependent on extracellular [Ca^{++}], for other biologically active peptides in the enteric nervous system an alternative mechanism of release can not be discounted. Since more than one mechanism is possible, the present studies must be regarded as preliminary in nature. Additional studies are necessary to reveal the release mechanism(s) of neuropeptides using isolated myenteric varicosities.

The trigger for the release of some neurotransmitters during

depolarization has been attributed to influx of Na^+ (Atwood et al., 1975; Baker & Crawford,1975) rather than [Ca^{++}] and subsequent mobilization of intracellular bound [Ca^{++}] (Schoffelmeer & Mulder, 1983) through [Na^+]/[Ca^{++}] exchange associated with intraterminal organelles (Sandoval,1980). In this regard, analysis of currents under whole-terminal voltage clamp of crustacean sinus gland peptidergic nerve terminals, showed inward currents carried by [Na^+] and by [Ca^{++}] (Lemos et al.,1986). A [Na^+] dependent release of GABA from nerve terminals that was independent of extracellular Ca^{++} has also been reported (Arias et al.,1984). In light of these reports with synaptosomes, it is conceivable that release of GalLI from enteric varicosities involves [Na^+] rather than [Ca^{++}] current to elevate internal [Ca^{++}] concentration, which in turn leads to GalLI release. In contrast, tachykinin release may involve both a calcium and a sodium current.

The [Ca^{++}]-fluorescent indicator fura-2 (Grynkiewicz et al., 1985; Brethes et al.,1987) which specifically measures stimulated changes in the $[Ca^{++}]_i$ of varicosities may be used to directly determine the calcium dependence of release of the neuropeptides identified in the isolated myenteric varicosities (GAL, ME, LE, VIP, SP, a-NK and GRP). A link between calcium entry and the adenosine receptor may be provided in studies correlating the effects of nucleosides on both endogenous peptide release and $[Ca^{++}]_i$ in varicosities.

A 3 min. reaction time was allowed for K^+ to evoke release of the neuropeptide entities from synaptosomes. Neurons releasing various messenger substances have been shown to exhibit large differences in the extent to which release can be sustained in the presence of elevated [K^+]: eg. 10-30 min. for peptides from pituitary neural lobe (Muller et al.,1975; Nordman, 1976); 1-2 min for catecholamines (Baker & Rink,1975) and for protein from pituitary intermediate lobe (Thornton,1982); and 5-10 min for amines from lobster pericardial organs (Cooke & Sullivan, 1982). On the basis of such reports, it is clear that the reaction time was probably sufficient to evoke release of neuropeptide entities from enteric varicosities.

The threshold K^+ concentration for causing release of neuropeptide-like-immunoreactivities from isolated varicosities varies between experiments for the same or different conditions. Further, the depolarization induced release of peptides appears to be inversely related to the basal release of each peptide. Thus, depolarization induced release of neuropeptides from varicosities is rather unpredictable. It is concluded that the isolated myenteric varicosity preparation is not a suitable model system for the study of nucleoside modulation of depolarization-induced release of peptide-like immunoreactivities. Perhaps a more useful approach would be to study modulation of receptor-mediated release of peptides.

Receptor-mediated release of neuropeptides from enteric nerve endings.

Release of SPLI, a-NKLI, LELI and GalLI by GRP from enteric nerve endings occurs in a concentration-dependent manner and this novel finding supports previous suggestions that GRP may function as an excitatory neurotransmitter in the enteric nervous system. Specifically, GRP appears to be involved in presynaptic modulation of neuropeptide release from enteric nerve endings (For a discussion of synaptic actions of putative transmitters on target cells, refer to Koketsu (1984)). Interestingly, release of both excitatory (SP & a-NK) and inhibitory mediators (GalLI & LELI) occurs with GRP. Whether these neuropeptide-immunoreactive entities are co-released, co-packaged or are involved in co-transmission is not known. The ability to detect release with GRP, of several neuropeptide entities, indicates that a substantial releasable pool of these entities remains in the varicosities following their isolation. As well, it is clear that the same isolation/resuspension conditions are adequate to study stimulated release of a variety of unrelated neuropeptide-immunoreactivities. It is worth noting that maximal responses to the agonists were not attained since the amounts of peptide required were not economically feasible. As such, any reference to potency refers to the ability of the agonists to evoke release of peptide-immunoreactivity at comparable doses on a concentration response curve.

The lower potency of bombesin observed in comparison to that for GRP in releasing SP- and a-NK-like immunoreactivities from enteric nerve endings, is paralleled by its potency in causing TTX-sensitive contractions of the more intact guinea pig LMMP preparation. Such parallel findings support a receptor-mediated interaction for GRP with isolated myenteric varicosities.

The GRP receptor antagonist [Leu^{13}- -CH_2NH-Leu^{14}] bombesin failed to shift the dose-effect curves to GRP and bombesin at releasing tachykinins from P_2 synaptosomes. Given the high doses of GRP and bombesin required to cause release of TK's it is likely that the concentration of antagonist (50uM) included in the incubation mixture was insufficient to compete for receptor sites with the agonists. This antagonist has been reported to be a potent antagonist of ^{125}I-labelled [Tyr^4]bombesin binding in acinar cells (Coy,1987). In other experiments, inclusion of the opioid receptor antagonist in the incubation mixture did not alter the responses to GRP on releasing the tachykinins and this may be taken to suggest that endogenous opioid substances (eg. high basal release of MELI) do not exert an inhibitory influence on the release of TK's in this system.

Evidence for significant differences in the size of the releasable pools of TK's has been obtained using GRP as a releasing agent. GRP was shown to release more than 3 times the amount of a-NKLI than SPLI from the same preparations, whereas the neuronal contents for

a-NK- and SP- LI's in P_2 lysates was in the ratio of approximately 1:1. From these findings it may be suggested that either a-NK is co-packaged in the same releasable pools as SP in a ratio of 3:1 or different releasable pools exist for each TK in the same or different sub-populations of varicosities. In either case a-NK appears to be different than SP in this respect. An alternative explanation is that released SPLI is degraded faster than a-NKLI. Comparison of the rates of degradation of exogenous SP and a-NK by enzymes present in the varicosity suspension would provide an answer to this question.

The rank order of potency of GRP and bombesin in releasing GALLI was reversed (Bombesin > GRP) from that which was found for the previously discussed peptides. The reversal in potency may be explained simply on the basis of differences in the maximal attainable release of the neuropeptides. A true potency measure refers to the EC_{50} value of a dose-response relationship which achieves a maximum plateau response. Given that such maximal responses were not achieved with GRP and bombesin, depending on the steepness of the full dose-response relationships, the potency ratio may be expected to vary considerably.

When looking at the evokable release of neuropeptides from this heterogeneous population of enteric nerve endings, it is important to consider the basal release (spontaneous release) of other neuropeptides present in the preparation. To what extent these

endogenous substances influence the release of each other is not understood. Neuropeptide receptors easily undergo tachyphylaxis and /or desensitization. Consequently, as a result of the continuous basal release of certain peptides from varicosities, it may be expected that relatively higher concentrations of exogenously applied neuropeptides would be required for effect. This may explain the apparently high concentration of GRP and bombesin required to release neuropeptides. In extreme situations, peptides may appear to be without effect. Rapid degradation of exogenously applied GRP, by enzymes present in the synaptosomal suspension, could also account for the high concentrations required. Some damage of the GRP receptors present on varicosities during the isolation procedure could also be a contributing factor.

In preliminary experiments, the effect of the A_1-selective adenosine receptor agonist cyclopentyladenosine on basal and GRP-mediated release of the tachykinins was investigated. These studies failed to show inhibition of the release of tachykinin-like immunoreactivity by the nucleoside analog (100uM). Speculation on the reason for the apparent lack of effect of CPA at this point would be premature.

SUMMARY AND CONCLUSIONS

1. Isolation of partially purified myenteric varicosities yielded preparations that were clearly enriched with spontaneously resealed varicosities as indicated by both the occluded LDH activity and the morphology of PV suspensions revealed in electron microscopic studies. Morphological studies illustrated the heterogeneous nature of the PV preparation. Quantitation of the neuronal contents of several neuropeptide-immunoreactivities clearly demonstrated the heterogeneity of peptidergic nerve terminals in the PV.

2. The binding of [^{3}H]-NECA and [^{3}H]-CHA at PV was saturable and the binding isotherms yielded affinities which were closely related to the concentration range over which these nucleosides cause inhibition of transmitter release in the stimulated guinea pig ileal preparation. Their equilibrium dissociation constants (K_d values) correlate well with their respective EC_{50} values obtained in the functional system, and the number of binding sites (Bmax) labeled by these nucleosides ($1.5\pm.47$ pmoles/mg and $.34\pm.19$ pmoles/mg respectively) were consistent with low capacity binding observed for receptors.

3. The saturation binding characteristics of [^{3}H]-NECA and [^{3}H]-CHA at PV strongly suggest that the binding sites for these nucleosides represent the biologically relevant adenosine receptor(s) located on myenteric nerve endings of the small intestine.

4. Since both the mixed A_1/A_2 agonist NECA and the selective A_1 agonist CHA share the same K_d value, these receptors are not classifiable according to the current criteria (Stone,1985) into A_1/A_2 subtypes. [^{3}H]-NECA also binds to a population of binding sites not recognized by [^{3}H]-CHA, suggesting that an A_2-receptor subtype may also be revealed by NECA.

5. Binding to the mitochondrial fraction (MITF) by labeled NECA or CHA was also saturable and had similar K_d values to those obtained at the PV. The number of binding sites labeled by NECA were the same as those labeled at PV, whereas the number of binding sites for CHA at MITF was significantly higher than at PV. It is likely that some binding of [^{3}H]-NECA to MITF represents binding to 'atypical' adenosine receptors on varicosity membranes contaminating the MITF. Alternatively, this site represents the A_2 receptor on smooth muscle membranes and/or varicosities described by Gustaffson (1985).

6. [^{3}H]-CHA] and [^{3}H]-NECA binding to PV have lower affinity and higher capacity than those obtained at brain membranes. It is concluded that the adenosine receptor(s) at PV which is recognized by both CHA and NECA is different from that described in brain. This receptor may be an A_3 type receptor.

7. In binding competition experiments at PV, R-PIA, CHA and NECA displaced equimolar concentrations of their tritium-labeled

counterparts with similar competition profiles and IC_{50} values. A potency profile of CHA = NECA = R-PIA did not permit subclassification of these binding sites into A_1/A_2 subtypes.

8. Displacement of the A_1-selective ligand [^{3}H]-CHA by unlabeled CHA and by the mixed A_1/A_2 ligand NECA generated similar displacement profiles with comparable IC_{50} values. In contrast, the potency order of selective adenosine analogs as displacers of [^{3}H]-NECA was NECA = 2CADO > R-PIA = CHA = CPA = R-PBA. These findings support the existence of two distinct binding sites at PV, with one binding site only recognized by the A_2 ligand NECA and by 2CADO. The ability of 2CADO to displace the residual binding of [^{3}H]-NECA strongly supports the existence of an A_2 adenosine receptor at PV.

9. The A_1-selective adenosine receptor antagonist DPSPX was a more effective displacer of the binding of [^{3}H]-CHA than it was of [^{3}H]-NECA. The data is consistent with heterogeneity of adenosine receptor sites on PV and provides further support for the existence of an A_2 adenosine receptor at this locus.

10. Competition experiments with [^{3}H]-NECA and [^{3}H]-R-PIA established the relative affinities of several unlabeled nucleoside analogs for binding sites at PV. The potency order for these analogs was R-PIA = 2CADO > ADO-N^1-OX > PUR RIB >= INO as displacers of binding of [^{3}H]-R-PIA and NECA = 2CADO > ADO-N^1-OX > PUR RIB >= INO as displacers of binding of [^{3}H]-NECA. Such potency profiles

established the structural specificity of [^{3}H]-NECA and [^{3}H]-R-PIA binding sites for nucleoside analogs.

11. An excellent correlation with unit slope ($p > .05$) was obtained between the relative binding affinities of nucleoside analogs for labeled-NECA and R-PIA binding at PV and their potency as inhibitors of Ach release from the stimulated ileum preparation.
The potency of adenosine receptor antagonists (eg. theophylline and DPSPX) as displacers of [^{3}H]-NECA, [^{3}H]-R-PIA or [^{3}H]-CHA binding at PV was paralleled by their relative pA_2 values obtained in the functional system. Relative affinities of selective adenosine receptor agonists (eg. NECA, CHA and R-PIA) for binding sites at PV (determined by displacement of their labeled counterparts) were found to be similar to their relative potencies as inhibitors of Ach release in the functional system. It is concluded that the binding sites labeled by [^{3}H]-NECA and [^{3}H]-CHA and [^{3}H]-R-PIA represent biologically relevant adenosine receptors in the enteric nervous system.

12. The rank order of potencies of adenosine receptor analogs as inhibitors of Ach release from the stimulated guinea pig ileum (ES, .2Hz, 1msec pulse duration) was CPA = R-PBA > NECA = R-PIA = 2CADO >= MCPA > S-PIA > ADO. This potency profile does not allow subclassification of the peripheral adenosine receptor(s) into A_1/A_2 subtypes. These characteristics are more accurately defined by an A_3 adenosine receptor. The 10 fold greater potency of R-PIA over S-PIA

is consistent with an A_2-type interaction. However, this potency difference between diastereoisomers contrasts that obtained by others (Paton,1981; Gustaffson, 1985). The discrepancy may be attributed to the different stimulation parameters used. It was noted that some problems and controversy exist with using R/S differences to denote receptor subtypes. It is concluded that the peripheral adenosine receptor(s) revealed in functional studies is either 'atypical'or the criteria for defining such receptors are inappropriate.

13. The ability to characterize the interaction of antagonists with the adenosine receptor(s) present on myenteric nerve terminals of the guinea pig ileum and thus to obtain the affinities of the antagonists, permitted possible heterogeneity to be revealed in a functional system. Results with the antagonist theophylline (THEO) clearly demonstrated the competitive nature of the antagonism. The affinity of THEO for the receptor(s) (pA_2) against the mixed agonist NECA was comparable to that obtained in the presence of several A_1-selective agonists. These data support the existence of a unitary adenosine receptor at the peripheral locus. The lack of A_1/A_2 selectivity of THEO did not allow any possible adenosine receptor heterogeneity to be revealed.

14. In Schild analysis, the A_1-selective adenosine receptor antagonist DPSPX yielded comparable pA_2 values in the presence of several A_1-selective adenosine receptor agonists and the interaction

of DPSPX with the receptor(s) was competitive. Such data was also consistent with the presence of a unitary adenosine receptor. In contrast, Schild data obtained for DPSPX in the presence of the mixed A_1/A_2 agonist NECA was best described by a curvilinear isobole. Deviations of the Schild isobole of this type are consistent with those observed on theoretical grounds for a heterogeneous population of receptors having unequal receptor concentrations. Thus, it is suggested that prejunctional neurotransmission in the ileum may be modulated by nucleosides via both an 'atypical' receptor (i.e. possibly an A_3-like receptor) as well as an A_2 adenosine receptor. The possibility exists that NECA interacts with postjunctional A_2 adenosine receptors on smooth muscle at the higher doses needed to inhibit Ach release in the presence of high doses of DPSPX.

15. Adenosine-mediated regulation of tachykinin release from myenteric nerves was studied using the atropinized guinea pig LMMP. Equieffective responses to CCK8 (5nM) and electrical stimulation (ES, 20Hz, 0.75msec pulse duration) were used in the study of tachykinin release. These TTX-sensitive responses were mediated by tachykinins since desensitization with substance P (SP) or the presence of the SP antagonist [d-Pro^4,Trp^{7,9,10}] SP (4-11) in the bathing medium almost abolished these responses.

16. Selective adenosine receptor agonists dose-dependently inhibited the release of tachykinins which was mediated by either ES or CCK8.

These novel findings strongly suggested that nucleosides may regulate the release of tachykinins from myenteric nerves.

17. Nucleoside inhibition of the CCK8-mediated release of tachykinins revealed the potency profile of CPA > NECA > 2PAA, suggesting that the adenosine receptor(s) mediating such inhibition is similar to that involved in modulation of cholinergic transmission. In contrast, the lack of difference between the potencies of CPA and NECA (i.e. CPA = NECA > 2PAA) obtained for ES-mediated responses may indicate the existence of a distinctly different adenosine receptor which modulates the release of tachykinins. This possibility is consistent with the 780 fold selectivity of CPA for A_1 sites in comparison to A_2 sites (Phillis,1985). The existence of different adenosine receptors on two distinct populations of varicosities, one of which contains tachykinins, must thus be contemplated.

18. The inability of selective nucleoside analogs to inhibit 100 percent of the TTX-sensitive ES-responses of the atropinized LMMP suggests that nucleosides do not inhibit all excitatory mediators released on ES.

19. From reverse-phase HPLC analysis, it was evident that concentrated preparations of varicosities (PV), derived from the guinea pig LMMP, contain substantial quantities of the tachykinins a-neurokinin and substance P-like immunoreactivities (LI's), whereas

b-neurokinin-LI was usually undetectable. It was concluded that adenosine or ATP regulate only the release of a-neurokinin and/or substance P from myenteric nerves of the guinea pig ileum, since b-neurokinin is not likely to contribute to the contractions produced by either ES or CCK8.

20. The data obtained from Schild analysis, indicated that CPDPX and DPSPX are competitive antagonists at adenosine receptors mediating inhibition of the CCK8-mediated release of tachykinins. The higher affinity displayed by CPDPX over DPSPX is consistent with its higher affinity for A_1 adenosine receptors. Therefore, inhibition of the release of tachykinins by CCK8 is mediated by an adenosine receptor possessing some A_1-like characteristics. The comparable pA_2 values obtained in the presence of NECA and CPA did not allow receptor heterogeneity to be revealed.

21. Adenosine may modulate the release of both ACh and the CCK8-mediated release of tachykinins via similar adenosine receptor(s). A different adenosine receptor may regulate ES-induced release of tachykinins from myenteric nerves. These latter receptors are likely to be located on separate tachykinergic neurons.

22. Characterization of the isolated myenteric nerve ending preparations (PV and P_2) was carried out in order to assess suitability as a model system for the study of modulation of release of endogenous neuropeptide-LI's by nucleosides. RIA dose-inhibition

relations of SPLI, VIPLI, GRPLI, LELI, MELI and GALLI in PV produced dose-inhibition curves close to or parallel with those produced by their respective synthetic peptides. These studies served to validate the RIA systems employed. Reverse-phase HPLC analysis allowed the identification of the molecular forms of the peptide-like immunoreactivities present in PV. The inability of sensitive RIA systems to detect either gastrin- or PYY-LI in PV is consistent with no contamination of the PV by endocrine elements.

23. The concentration of peptide-LI's measured in PV are in agreement with previous immunohistochemical and tissue extract studies carried out on small intestinal tissue. The concentration profile obtained is SPLI > MELI > LELI > VIPLI > GRPLI > Galanin. The relative amounts of peptide-LI's may not accurately reflect the in vivo concentrations since differing rates of depletion of varicosities may occur during isolation.

24. Reverse-phase chromatography of the PV preparations indicated that guinea pig SPLI has close identity to, or is structurally identical with, SP from another species. A major peak of SPLI was also detected which had a considerably different retention time than any other tachykinins, extended forms or their products. The nature of this peak is at present unclear. A small but significant peak of SPLI occured at the identical retention times of a-NK suggesting that this tachykinin is present in myenteric plexus and is structurally similar to its porcine counterpart (note: the SP

antiserum has 0.3% cross-reactivity with a-NK). Using an antiserum for a-NK (with 50% cross-reactivity for b-neurokinin), equal amounts of a-NKLI and SPLI were measured in P_2. It is concluded that the newly identified mammalian tachykinin a-NK may also be a peptide with a physiological role in the enteric nervous system. As no b-neurokinin immunoreactivity was detected with reverse-phase HPLC, its physiological significance is unclear.

25. LE and ME-LI in the PV preparation eluted at retention times similar to their synthetic analogs, galanin-LI eluted at a retention time different from that of synthetic porcine galanin and VIPLI eluted at a retention time of synthetic guinea pig VIP. GRPLI eluted a retention time close to that of synthetic porcine GRP (1-27) and its major oxidized form.

26. Identification of substantial quantities of several peptide-immunoreactivities in the isolated myenteric varicosity preparation (PV) suggested that this preparation may prove useful in release studies involving neuropeptides. The prominence of a-NK in the PV and P_2 may allow a re-evaluation of the nature and function of previously documented 'SPLI' pathways present in mammalian gut, using these preparations.

27. The relative distribution of peptide-like immunoreactivity and occluded LDH activity was determined in various fractions obtained by differential centrifugation of homogenates of guinea pig ileal

LMMP. The relative distribution of occluded LDH activity indicated that the highest concentration of varicosities was present in the P_2, with considerably lower amounts found in fractions F_a, F_b and F_c obtained from continuous sucrose gradients. Similarly, the highest concentrations of GRP, VIP, ME and LE-LI's were present in the P_2 fraction. The inappropriate V_m of the PV determined by the TPP^+ cation method together with these findings, imply that the P_2 may be the more appropriate fraction for carrying out release studies.

28. Recovery of LE, VIP and GRP from continuous gradients was about 50% of P_2 neuronal content. In contrast, only 10% of MELI was recovered in these fractions. It is likely that the higher basal release of MELI, compared to other peptides observed in the P_2, results in extensive depletion of the neuronal content of MELI.

29.The guinea pig enteric nerve ending preparation (P_2/PV) is a rich source of degradative enzymes as indicated by its rapid degradation of exogenous SP. Bacitracin alone effectively inhibited degradation of exogenous SP. This protease inhibitor also retarded degradation of endogenously released SP as demonstrated by the enhanced basal release of SPLI from P_2. Considerable depletion of neuronal content of SPLI was also noted in the P_2 that could be explained by its high basal release.

30. Significant differences in the basal release (pmoles/mg/min) exist between neuropeptide-like immunoreactivities. The rate of

release following a 60 min equilibration of the P_2 was highest for MELI, followed, in descending order by LELI > a-NKLI = SPLI. Such basal release may be involved in significant interactions between varicosities. An accurate determination of the time course of the basal release of neuropeptides may be needed in order to study evokable release of peptides, as indicated by a time-dependent decrease in basal release of SPLI.

31. The basal release of LELI, a-NKLI and SPLI may involve a calcium dependent mechanism, since D600 blocked about 50% of their basal release. The data suggests that the varicosities may be partially depolarized since D600 blocks influx of calcium through open voltage sensitive channels. As a consequence, it would be more difficult to further depolarize synaptosomes with elevated K^+. Depolarization-induced release of a-neurokinin-, met-enk- and substance P-LI's appeared to be inversely related to their basal release. Such findings suggest that basal release of neuropeptides may serve as a marker for the relative depolarization state of the varicosities. About 25% of the remaining basal release of a-NKLI and SPLI may be attributed to the low centrifugation speed used to terminate the release reaction. It is concluded that a portion of the basal release may represent a functional release which may be subject to further modulation by exogenous agents.

32. The Na^+ ionophore monensin dose-dependently evoked release of a-NK and SPLI from P_2 and relatively more a-NK was released han SPLI.

The calcium ionophore A23187 or the stimulator of adenylate cyclase forskolin, failed to evoke detectable release of the tachykinins. Such data suggests that influx of Na^+ may cause release of a substantial pool of tachykinins from myenteric nerve endings.

33. A clear dose-dependent release of a-NKLI, MELI and LELI was observed with the depolarizing stimulus K^+. These findings suggest that the P_2 contains entities with an appropriate V_m and that they can undergo depolarization leading to an exocytotic release of neuropeptide-immunoreactivities. The data provides novel evidence which suggests that the newly identified mammalian tachykinin a-neurokinin as well as LE may function as neuromediators in the enteric nervous system.

34. Release of neuropeptide-LI was dependent on the antagonists included in the P_2 incubation mixture. Release of MELI with elevated K^+ was clearly evident only in the presence of either atropine or naloxone. In contrast, release of LELI was clearly evident only when atropine, naloxone, and the A_1 adenosine receptor antagonist DPSPX were present. It is concluded, that such differences in antagonist requirements, are consistent with the existence of distinct populations of varicosities containing either LELI or MELI, the release of which may be under differential regulation.

35. The depolarizing stimulus K^+ was unable to cause detectable release of the tachykinin SPLI, in the same experiments in which it

caused release of MELI and LELI from P_2. These data, in conjunction with previous findings, may support the existence of distinct populations of nerve endings containing SPLI, LELI or MELI, in keeping with immunohistochemical data.

36.Clear depolarization-induced release of a-NKLI from P_2 was evident in the presence, but not the absence, of the A_1-selective adenosine receptor antagonist DPSPX. A possible explanation for the data is that endogenous adenosine may be involved in the neuroregulation of release of a-NKLI from varicosities. This interpretation is consistent with the existence of an A_3 adenosine receptor on enteric nerve endings.

37. The K^+-evoked release of a-NK (7% of neuronal content) is significantly lower than that observed for the enkephalins (30% of neuronal content). These findings support the view that, in these preparations, varicosities which contain MELI exhibit a different depolarization state than those containing a-neurokinin.

38. The threshold concentrations of K^+ required to evoke release of a-NKLI or MELI were different, suggesting that these neuropeptide entities may exist in separate populations of varicosities, set at different membrane potentials.

39. Depolarization-induced release of GALLI was not detectable from isolated myenteric nerve endings (P_2), in the same preparations in which release of other peptides was noted. Degradation and or the

low neuronal content may account for this lack of effect.

40. In general, the depolarization-induced release of neuropeptides from isolated myenteric varicosities was unpredictable. It is therefore concluded that the P_2 preparation is not a suitable model system for the study of nucleoside modulation of depolarization induced release of peptide-like immunoreactivities. It was felt that a more useful approach would be the study of receptor-mediated release of peptides from P_2.

41. Gastrin releasing polypeptide (GRP) was shown to dose-dependently evoke release of a-NKLI, SPLI and GALLI from P_2 varicosities. The lower potency of bombesin observed for the release of tachykinins from P_2 was paralleled by its lower potency in causing TTX sensitive contractions of the more intact atropinized LMMP. GRP was more potent at releasing a-NK and SP-LI's from P_2 following a 60 min rather than a 30 min equilibration at 37 C following resuspension. These findings support a receptor mediated interaction for GRP at varicosities.

42. GRP was shown to release approximately three times the amount of a-NKLI than SPLI from the same P_2 preparations. Such differences may reflect differences in the rates of degradation or location of releasable pools of the tachykinins.

43. The results with GRP appear promising and further experiments are warranted to determine the suitability of using 'GRP-mediated release of neuropeptides from P_2' as a model system to study nucleoside regulation of the release of endogenous peptide-LI's.

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