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Opioid Receptor Effects Of Two Aminotetralin Derivatives In Guinea Pig Ileum Longitudinal Muscle And Mouse Vas Deferens Preparations

Francisca Fatima Matos
University of the Pacific

OPIOID RECEPTOR EFFECTS OF TWO AMINOTETRALIN DERIVATIVES IN GUINEA PIG ILEUM LONGITUDINAL MUSCLE AND MOUSE VAS DEFERENS PREPARATIONS

by

Francisca Fátima Matos, M.S.

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This dissertation, written and submitted by

Francisca Fátima Matos

is approved for recommendation to the Committee on Graduate Studies, University of the Pacific

Dean of the School or Department Chair:

Donald Y. Shirachi

Dissertation Committee:

Chair: Mary H. Mahe

Co-Chair: Kathryn A. Lambert, Ph.D.

David L. Fenner James W. Blankenship

Horace H. Loh

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ABSTRACT

Two substituted analogs of 3-amino-2,2-dimethyltetralin, namely 3-dimethylamino-7-hydroxy-2,2-dimethyl-1-tetralone HBr (J) and 3-dimethylamino-7-hydroxy-2,2-dimethyl-1-tetralol (MRSAL) were evaluated for opioid agonist and antagonist activity using the electrically-stimulated guinea pig ileum longitudinal muscle and mouse vas deferens preparations. The effects of these compounds in these tissues were compared to those induced by several opioid prototype agonists at mu, kappa and delta sites (normorphine, dihydromorphine, ethylketocyclazocine, U-50,488H, beta-endorphin, dynorphin 1-13, leu-enkephalin and DADLE) and one opioid antagonist (naloxone).

The results of these experiments demonstrated that compound J inhibited contractions in a concentration-dependent manner as an opioid agonist and its effects were antagonized by naloxone in both preparations. The agonist effects of J were also irreversibly antagonized by beta-funaltrexamine pretreatment suggesting a preference for mu receptors. On the other hand, MRSAL was able to antagonize all the opioid agonist prototypes in a concentration-dependent manner, but with varying affinities.

The differential opioid receptor selectivity for compound J was studied based on: (i) its agonist potency in guinea pig ileum longitudinal muscle versus the mouse vas deferens; (ii) its irreversible antagonism by beta-funaltrexamine in guinea pig ileum; and (iii) by the

calculation of the apparent dissociation constant (Ke) of naloxone for this agonist in both preparations. The opioid receptor preference for MRSAL was based on its potency in antagonizing the opioid agonist prototypes by calculating its Ke value. Based on these criteria, compound J behaved as a mu receptor agonist while MRSAL had preference for mu rather than kappa or delta receptors.

"We pharmacologists must acquire
a knowledge of the tools which
we use."

R. Buchheim, 1849

I dedicate this dissertation to

my mother, Francisca Castelo Matos,

who has shown me to be strong

willed and to persevere.

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INTRODUCTION

Pain is probably the most primitive and most elemental physical and emotional distress experienced by animals and humans. Teleologically, pain functions as an extremely important warning system to alert an individual that something is wrong and that a state of trauma or disease exists. When pain becomes constant, intense and intolerable, it is debilitating and both its clinical assessment and management become necessary. To date, drug therapy in the management of pain can be divided into two major categories: Group I, the nonsteroidal anti-inflammatory drugs and nonnarcotic analgesics for the treatment of minor to moderate pain, and Group II, the narcotic analgesic drugs for the treatment of intense pain. The first group of drugs (aspirin, phenylbutazone, acetaminophen, ibuprofen, naproxen, fenoprofen, meclofenamate, indomethacin, sulindac, zomepirac) act peripherally and probably produce analgesia by blocking or reducing the production of the various autacoids that activate peripheral pain sensors (Brune and Lanz, 1984; Kantor, 1984). The second group of drugs (morphine alkaloids and synthetic analogs) appear to produce analgesia by interacting with stereospecific opioid receptors found in the central nervous system and associated with perception of pain (Chang and Cuatrecasas, 1979; Simon, 1979; Zukin, 1984).

Morphine, the major alkaloid present in the latex from unripe seed capsules of the opium poppy, Papaver somniferum, was isolated by the

German pharmacist Setürner in 1803 (Casy and Parfitt, 1986) and has been used as an analgesic since the nineteenth century. However, morphine induces serious side effects, such as smooth muscle spasm and respiratory depression, in addition to tolerance and physical dependence (Inturrisi and Foley, 1984) in animals and humans. At the present time, morphine is not synthesized commercially so its production continues to be dependent on the growth of the opium poppy. Therefore, there has been an intensive search for a synthetic non-addictive morphine substitute that would produce minimal tolerance and adverse side effects. Many chemicals, most structurally similar to morphine, have been synthesized but, to date, no single compound is clearly superior to morphine considering all of the pharmacologic and toxicologic aspects. Nevertheless, many of these synthetic analgesics are available commercially or for research use, e.g., the benzomorphans, meperidine and related 4-phenylpiperidines (Fries, 1978; Michne, 1984), fentanyl and the 4-anilinopiperidines, methadone and related 3,3-diphenylpropylamines, benzimidazoles, tetrahydrioisoquinoline derivatives and the aminotetralins (Casy and Parfitt, 1986).

Historical Background of 2-Aminotetralins

The aminotetralins are considered to be structural A,B ring analogs of morphine (Fries and Bertelli, 1981) and structurally they are also similar to cyclazocine (Figure 1). The basic structural requirements for a potent morphine-like agonist are retained in the 2-aminotetralin skeleton. These minimum requirements are a tertiary amine, an alpha-quaternary carbon, a phenyl group connected to the quaternary carbon and a 2-carbon chain separating the tertiary nitrogen and the quaternary

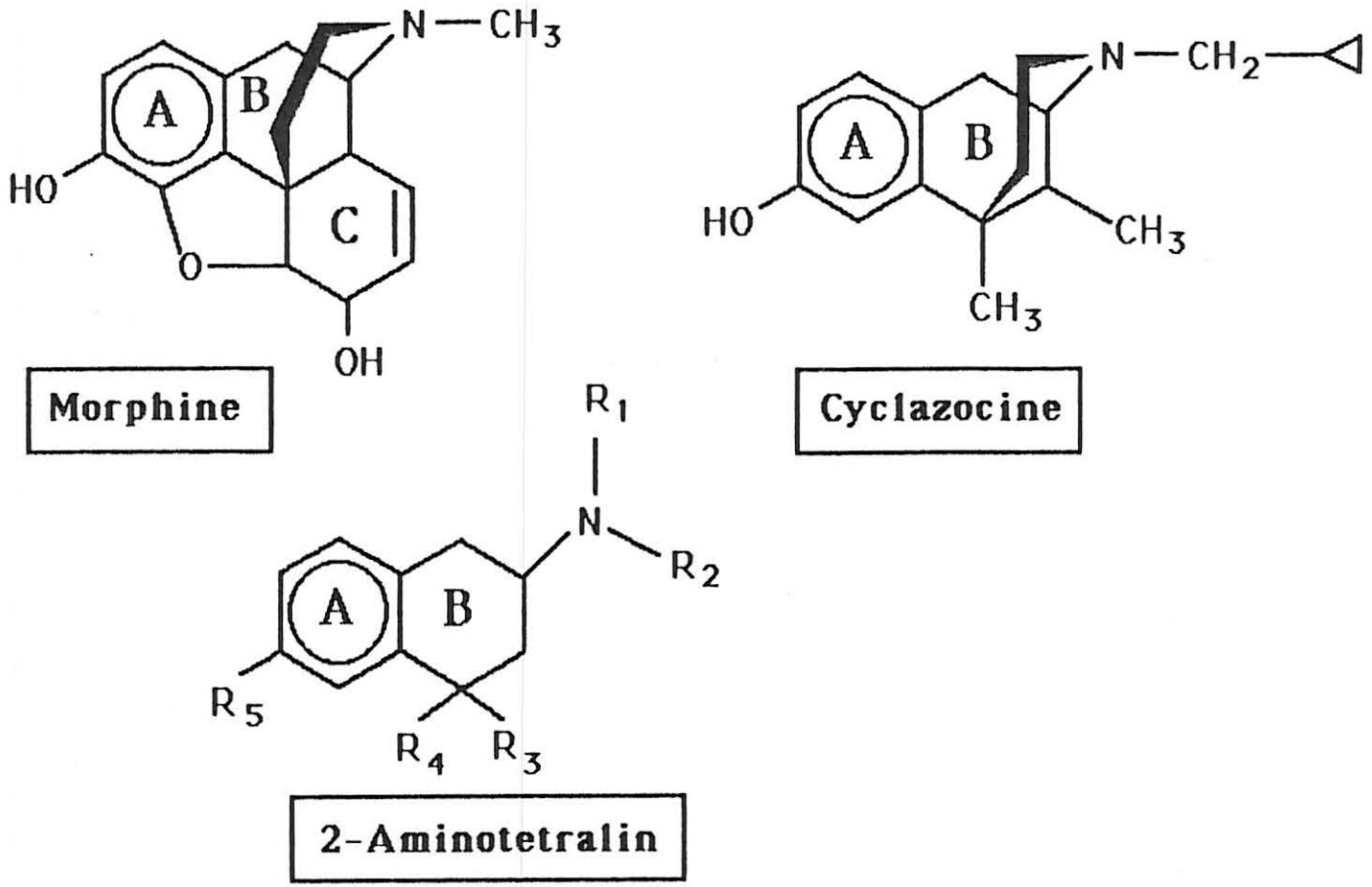


Figure 1. Chemical Structures of Morphine, Cyclazocine and 2-Aminotetralins.

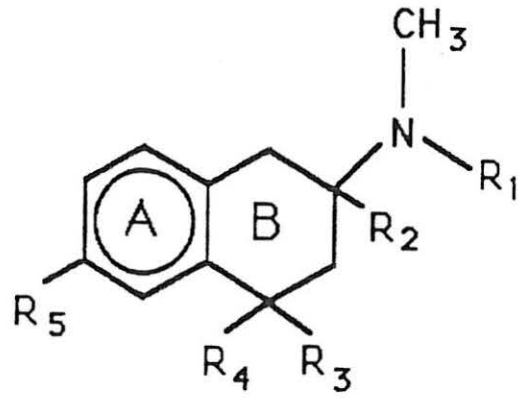
carbon (Figure 1) (Martin et al., 1969). Compounds containing the aminotetralin structure also have the potential for opioid antagonist activity.

The history of opioid-like aminotetralins begins with the work of Martin et al. (1969) and Kandeel and Martin (1973) when these investigators reported several aminotetralins with analgesic activity. These compounds were tested for analgesic capacity utilizing the widely used mouse hot-plate method (Eddy and Leimbach, 1953). The most potent compounds synthesized by Martin and his group (Martin et al., 1969; Pai et al., 1971; Kandeel and Martin, 1973) are shown in Table I. All of these compounds were less potent than morphine but roughly equivalent in potency to meperidine. Compounds IX and 4b were slightly more potent than meperidine.

A few years later, Reifenrath and Fries (1979) and Fries and Bertelli (1982) reported aminotetralins with mixed opioid agonist and antagonist activity (Table II). Compound 4c displayed only agonist activity, but was much less potent than morphine and codeine while compounds 13a and 13b demonstrated mixed agonist-antagonist properties. As antagonists, these two compounds (3 mg/Kg intraperitoneally) were able to cause 100% inhibition of the analgesic effect of morphine using the rat tail-flick assay (D'Amour and Smith, 1941). In addition, compound 4b displayed only opioid antagonist capacity but was much less potent than naloxone (4b = 33.5 mg/Kg, naloxone = 0.031 mg/Kg).

Unfortunately, very little pharmacological data are available on the potency and receptor specificity of these compounds since there have been no antagonism studies reported using naloxone (NX) or some other opioid antagonist.

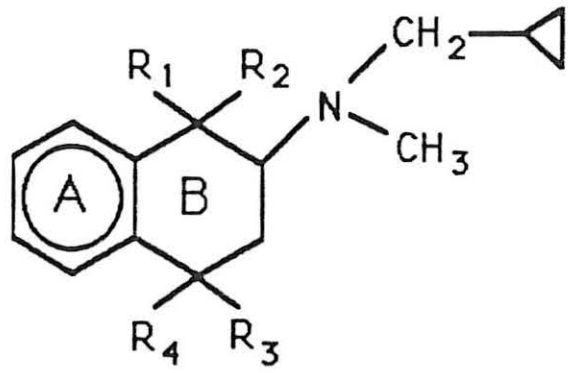
Table I. Analgesic Potency of Substituted 2-Aminotetralin Using Mouse Hot Plate Assay.



Compound	R_1	R_2	R_3	R_4	R_5	ED_{50}[1]
IX	CH_3	H	CH_3	CH_3	H	0.02
XII	H	$CH_2C_6H_5$	CH_3	CH_3	H	0.06
XIII	CH_3	CH_3	CH_3	CH_3	H	0.05
XIV	CH_3	$CH_2C_6H_5$	CH_3	CH_3	H	0.06
4a	CH_3	H	C_6H_5	CH_3	OH	0.04
4b	CH_3	H	CH_3	C_6H_5	OH	0.03
Meperidine						0.05
Morphine						0.005

[1]Median analgesic activity expressed as mmoles/Kg by intraperitoneal route.

Table II. Analgesic and Antagonist Potencies of Substituted 2-Aminotetralin.



Compound	R_1	R_2	R_3	R_4	ED_{50}[1]	AD_{50}[2]
4b	Ph	OMe	H	H	Inactive	33.5(16.1-69.7)
4c	Ph	OOOCEt	H	H	17.8 (10.9-29.0)	Inactive
13a	H	H	Ph	H	9.1 (5.2-15.9)	100[3]
13b	H	H	H	Ph	6.3 (4.3- 9.2)	100[3]
Morphine					1.1 (0.8- 1.5)	-
Codeine					7.5 (--[4])	-
Naloxone					-	0.031(0.01-0.09)

[1]Analgesic ED_{50} (mg/Kg) and its 95% confidence interval using mouse hot plate assay.
[2]Antagonist AD_{50} (mg/Kg) and its 95% confidence interval using mouse tail-flick assay for antagonism of morphine (6.5 mg/Kg).
[3]Percent inhibition (3 mg/Kg) shown after the administration of the ED_{50} of morphine using mouse tail-flick assay.
[4]Not reported.

Bridged aminotetralins are structurally similar to morphine and the benzomorphans. Dezocine, an example of a bridged aminotetralin, has been evaluated clinically (parenterally) and has been shown to have an analgesic potency in the morphine range with the usual side effects of opioids (Casy and Parfitt, 1986). As an opioid analgesic, this compound has an unusual pharmacologic profile in animals since it is devoid of agonist effects on the guinea pig ileum (Casy and Parfitt, 1986). In contrast, Greenberg and Ippersiel (1979) reported that dezocine (100 nM) effectively inhibited contractions antagonized by NX (100 nM) in this preparation. While it acts as an opioid agonist in drug-naive animals, it acts as an antagonist in morphine-dependent animals and precipitates withdrawal symptoms (Casy and Parfitt, 1986).

The aminotetralins are able to interact with a number of other pharmacological receptors. These other actions include: (i) marked dopamine agonist activity in the cat cardioaccelerator nerve assay (5-hydroxy-6-methyl-2-aminotetralin derivatives; Cannon and Koble, 1980); (ii) a D-2 dopamine receptor agonist effect because of its ability to inhibit the release of prolactin from dispersed anterior pituitary cells (N,N-disubstituted 2-aminotetralins; Beaulieu et al., 1984); (iii) a direct acting agonist capacity at post-junctional $alpha_1$-adrenergic receptors producing vasoconstriction in the isolated rabbit ear artery preparation (substituted 2-aminotetralins; DeMarinis et al., 1982); (iv) mixed adrenergic and dopaminergic agonist actions as well as a non-opioid analgesic activity not antagonized by naloxone in the mouse hot plate assay (2-aminotetralin derivatives; Cannon, et al., 1980); and (v) beta-adrenergic blocking actions in the isolated guinea pig atrial preparation (N-substituted 2-amino-6-hydroxy-1,2,3,4-tetrahydro-

napthalen-1-ol derivatives; Miyake et al., 1983).

One interesting derivative of 2-aminotetralin, namely N-methyl-N-(beta-phenylethyl)-1,2,3,4-tetrahydro-6-methoxy-4,4-dimethyl-2-naphthylamine HCl, has negative chronotropic and inotropic actions in addition to antifibrillatory properties in the isolated spontaneously-beating guinea pig atrial preparation (Johnson et al., 1970). Subsequently, Graeff et al. (1971) demonstrated that this compound has potent local anesthetic capacity but is devoid of beta-adrenergic blocking activity.

Opioid Receptors

During the past decade, several lines of evidence from pharmacological, biochemical and anatomical studies have accumulated, suggesting that there are multiple opioid receptors in the central nervous system and peripheral organs of animals and humans. It is now well accepted that mu (μ), kappa (κ) and delta (δ) opioid receptors exist. To a lesser extent, the epsilon (ϵ) and the sigma (σ) opioid receptors are also accepted.

Types of Opioid Receptors. The first evidence for the existence of multiple opioid receptors came from the pharmacological studies of Martin et al. (1976) who studied the effects of morphine- and nalorphine-like drugs in the chronic spinal dog. The authors postulated the existence of three stereochemically different opioid receptors based on three different syndromes mediated by these receptors: (i) the morphine syndrome (mu receptor) producing miosis, bradycardia, hypothermia, analgesia and indifference to environmental stimuli; (ii) the ketocyclazocine syndrome (kappa receptor) mediating miosis, sedation and

depression of the flexor reflex (analgesia); and (iii) the SKF-10,047 (N-allylnormetazocine) syndrome (sigma receptor) causing mydriasis, tachypnea, tachycardia and mania. All of these effects could be antagonized by naltrexone, an opioid antagonist, but each required a different dose level of naltrexone. Further studies demonstrated that chronic administration of morphine, ketocyclazocine and SKF-10,047 induced tolerance and qualitatively different abstinence syndromes, but only morphine was able to suppress the abstinence syndrome in morphine-dependent dogs. In vivo, kappa drugs can be distinguished from morphine-type drugs (mu) by their inability to suppress withdrawal syndrome in morphine-dependent monkeys (Martin et al., 1976; Lord et al. 1977). Martin (1981) has reviewed this work.

Subsequently, Lord et al. (1977) using isolated tissue preparations (mouse vas deferens and guinea pig ileum longitudinal muscle) extended the concept of multiple opioid receptors based on the different sensitivity of these two preparations to opioid agonists (normorphine, Met- and Leu-enkephalins and ethylketocyclazocine) and their sensitivity to naloxone antagonism. It was observed that the opioid receptor populations in these preparations were not identical since the mouse vas deferens was about eight times more sensitive to the enkephalins than the guinea pig ileum. On the other hand, the mouse vas deferens was less sensitive to the effects of morphine as compared to the guinea pig ileum. Furthermore, it was observed that the enkephalins in the guinea pig ileum were antagonized as well by naloxone as was normorphine. This suggested that in this preparation, both agonists interact with the same receptor population. However, in the mouse vas deferens it was necessary to use 10 times more naloxone to antagonize the effects of enkephalins

than for the antagonism of normorphine. This suggested that in this assay the enkephalins and normorphine are agonists at different receptors. Lord et al. (1977) concluded that, in the guinea pig ileum, enkephalins interact with the mu receptor of normorphine and morphine type agonists, but in the mouse vas deferens enkephalins act on a new receptor, the delta opioid receptor, while normorphine acts on the mu receptor. In addition, based on the sensitivity of naloxone antagonism against ethylketocyclazocine (3-6 times more naloxone is required to antagonize this agonist than mu-receptor agonists), Lord et al. (1977) independently postulated the existence of the kappa receptor originally proposed by Martin et al. (1976). In summary, the guinea pig ileum is said to have mu and kappa receptors while the mouse vas deferens has delta, mu and kappa opioid receptors.

Further pharmacological evidence for the presence of different opioid receptors in isolated tissues came from experiments using cross-tolerance with selective agonists for the different receptor populations (Wuster et al., 1981a,b; Schulz et al., 1981; Schulz et al., 1984). The development of tolerance by selective opioid receptor agonists has differentiated mu and kappa receptors in the guinea pig ileum and delta, mu and kappa receptors in the mouse vas deferens (Wuster et al., 1981b) but has failed to demonstrate delta receptors in the guinea pig ileum (Schulz et al., 1981). In these studies, selective mu agonists were fentanyl, normorphine and D-Ala^2,MePhe^4,Met(O)ol^5-enkephalin (DAGO), kappa agonists were 5,9-dimethyl-2'hydroxy-2-tetrahydroflurfuryl-6,7-benzomorphan (MR 2034), (5,9-dimethyl,2'5)-5,9-dimethyl-2'-hydroxy-2-(methoxy-propyl)-6,7-benzo- morphan (MRZ) and ethylketocyclazocine (EKC), and delta agonists were [D-Ala^2-D-Leu^5]-enkephalin (DADLE) and

Tyr-D-Ser-Gly-Phe-Leu-Thr (DSThr). The principle behind this technique is based on the assumption that if two agonists exhibit cross-tolerance, they must be acting at the same receptor site. Furthermore, if there is no cross-tolerance between two drugs, it means that their effects are produced by different receptor populations (Wuster et al., 1981a,b). In addition, using the cross-tolerance technique, Schulz and Wuster (1981) have postulated the existence of subtypes (isoreceptors) of mu and kappa receptors in the mouse vas deferens. In this experiment, sufentanyl and normorphine (both mu agonists) did not exhibit cross-tolerance to each other. MR 2034 and MRZ (kappa agonists) displayed cross-tolerance to ketocyclazocine and dynorphin, but not to each other. One, however, must be aware of the danger of receptor classification based on selectivity by exogenous compounds.

Pharmacologically active opioid receptors can also be discriminated from one another by using highly selective irreversible antagonists. Two potent opioid receptor alkylating agents have been used: beta-chlornaltrexamine (beta-CNA) and beta-funaltrexamine (beta-FNA)--both derivatives of naltrexone. Beta-CNA produces an irreversible antagonism that can be prevented, but not reversed, by naloxone at all opioid receptor types. It can block the effects of mu and kappa agonists in the guinea pig ileum and additionally the effects of delta agonists in the mouse vas deferens (Takemori and Portoghese, 1985a). The selectivity by which beta-CNA irreversibly blocks opioid receptors was mu > kappa > delta. Since beta-CNA irreversibly blocks all opioid receptors, it has been used for "protection" studies combined with an agonist with a selectivity for one of the opioid receptors. For example, in the mouse vas deferens, beta-CNA has been used with DSLET (an enkephalin analog

highly selective for delta receptors) to block irreversibly mu and kappa receptors without touching delta receptors (protected by DSLET). On the other hand, beta-FNA is a highly selective antagonist of mu receptors, blocking the effects of mu agonists in vitro and in vivo without affecting kappa or delta receptors (Takemori et al., 1981; Takemori and Portoghese, 1985a). Therefore, for example, beta-FNA has been used to deplete mu receptors in the guinea pig ileum, affording agonist access to the functional kappa receptors.

Other irreversible ligands available (for review see: Takemori and Portoghese, 1985a) to label different opioid receptors are: etonitazene isothiocyanate (BIT) (Rice et al., 1982) which labels mu receptors, fentanyl isothiocyanate (FIT) (Rothman et al., 1985) and fumaramido oripavine (FAO) (Rice et al., 1982) which label delta receptors, naloxazone and naloxonazine which label mu receptors (Goodman and Pasternak, 1984) and phenoxybenzamine which apparently has preference for mu receptors (Kosterlitz et al., 1981).

Recently, the heterogeneity of opioid receptors has been further extended by the demonstration that the rat vas deferens has epsilon receptors. These receptors are specific for beta-endorphin and (to a lesser extent) to enkephalins (Liao et al., 1981; Sanchez-Blazquez et al., 1984). In this preparation morphine acts as a partial agonist and is able to antagonize the inhibition of contractions induced by beta-endorphin (Liao, et al., 1981; Lord et al., 1982; Chang, 1984).

In summary, the opioid receptors pharmacologically found in various isolated tissue preparations are as follows: (i) guinea pig ileum, mu and kappa, (ii) mouse vas deferens, delta, mu and kappa; (iii) mouse and rat ileum, delta; (iv) rabbit ileum, delta and kappa; (v) dog ileum,

delta and mu; (vi) rat vas deferens, epsilon; and (vii) rabbit vas deferens, kappa (Oka et al., 1980a,b; Wuster et al., 1981b; Liao et al., 1981; Chang, 1984).

Biochemical evidence for opioid receptor multiplicity was only possible with the development of highly selective ligands for each class of receptor. Goldstein et al. (1971) was the first to describe a method to define stereospecificity for the opioid receptor, separating out the non-specific binding of ligand to structures such as brain membranes. Since then, mu, kappa and delta opioid binding sites have been characterized in the synaptosomal fraction of homogenate preparations from rat, mouse, and guinea pig brains (Lord et al., 1977; Kosterlitz et al., 1981; Chang et al., 1979), monkey and human brains (Lord et al., 1982), toad brain (Simon et al., 1982), as well as in guinea pig ileum (Creese and Snyder, 1975; Zukin, 1984). Guinea pig cerebellum has been shown to be rich in kappa receptors (Itzhak et al., 1984) and cultured mouse neuroblastoma or neuroblastoma-glioma hybrid cells have been shown to have a homogeneous population of delta receptors (Chang and Cuatrecasas, 1979; Chang, 1984). However, the adenylate cyclase of this latter system can be inhibited or dually regulated depending upon the time of exposure by the enkephalins, morphine and other opiate alkaloids (Sharma et al., 1977; Lord et al., 1982; Chang, 1984).

Opioid binding sites are stereospecific, saturable, associated with the membrane fraction of nervous tissue and demonstrate high affinity binding with both opioid agonists and antagonists. The binding sites have a pH optimum in the physiological range of 6.5 - 8.0 (Simon, 1979) and are influenced by temperature and sodium ions (100 mM) (Pert and Snyder, 1974; Lord et al., 1982). Interestingly, the effect of sodium

ions on opioid binding has been used to discriminate between agonist and antagonist binding since in the presence of sodium (100 mM), the binding of agonists is markedly reduced with little increase or no effect on the binding of antagonists (Lord et al., 1982; Pert and Snyder, 1974).

To date, several selective ligands have been used for demonstrating the presence of multiple opioid receptors in brain. In general, the order of selectivity of radioactively labeled opioids are as follows: mu receptor, morphiceptin, DAGO (an enkephalin analogue), fentanyl, FK 33824 (an enkephalin analogue), syndyphalin, morphine, dihydromorphine, normorphine, naloxone; kappa receptor, U-50,488H, dynorphin 1-13 and certain fragments of prodynorphin; delta receptor, DPDPE, DPLPE, DTLET, DADLE, DSLET (enkephalin analogues with agonist activity) and ICI 154129 (an enkephalin analogue with delta antagonist activity) (Chang, 1984; Paterson et al., 1984). A high degree of cross-reactivity for most labeled enkephalins with mu binding sites in whole brain preparations does occur. The same is observed with kappa agonists such as ketocyclazocine and ethylketocyclazocine. This latter agonist binds to both mu and delta sites with high affinity (Chang, 1984).

Recently, Pasternak (1986) and Goodman and Pasternak (1984) described a new high affinity site, mu_1, which is irreversibly antagonized by naloxazone and naloxonazine. In binding studies, both opiates and enkephalins bind with highest affinity (K_D < 1 nM) to this mu_1 common site. Once mu_1 sites are labeled with either one of the antagonists above, mu_2 sites are seen as a lower affinity site (K_D < 10 nM) which can now be labeled by dihydromorphine and other opiates and also the low affinity delta site can be labeled by DADLE. The physiological significance of these high affinity mu_1 sites seems to be

related to opioid analgesia. Since naloxazone treatment shifts the dose-response curve for analgesic effect of morphine, enkephalins and even beta-endorphin without affecting mu_2 sites under certain conditions, Pasternak (1986) concluded that mu_1 sites mediate opioid analgesia. These mu_1 sites appear to be involved in supraspinal analgesia, prolactin release, acetylcholine turnover, catalepsy and hypothermia, while spinal analgesia may be mediated by delta and/or kappa receptors. In addition, dopamine turnover and growth hormone release are mu_2 or delta, sedation is kappa while respiratory depression and inhibition of contractions of the guinea pig ileum are mu_2 mediated.

The sigma opioid receptor, originally proposed by Martin et al. (1976), is probably not a true opioid receptor but may be the receptor for phencyclidine (PCP). In binding studies, it was demonstrated that of all the opiates tested, only cyclazocine-like drugs were able to displace PCP from its binding sites and vice-versa. Behavioral studies also seem to demonstrate this "nonopioid" characteristic of cyclazocine. The psychotomimetic effects of cyclazocine are only partially reversed by naloxone, indicating that there is a nonopioid component to the effects of cyclazocine (Zukin and Zukin, 1981).

Localization of Opioid Receptors. Opioid receptors are distributed in specific brain areas of all mammals and probably all vertebrates; however, the distribution is variable among species. Binding in specific brain areas as well as the neuroanatomical localization of mu, kappa and delta opioid receptors have been well characterized and allow some correlation with pharmacological data. Autoradiographic and binding studies have detected opioid binding sites in brain and spinal cord.

Different ratios as well as different localizations exist for mu and delta receptors. It appears that mu binding is predominant in the thalamus, hypothalamus, the periaqueductal gray, interpeduncular nucleus, inferior colliculus, median raphe nucleus, limbic cortex and in the laminae I and IV of cerebral cortex. On the other hand, delta sites occur in the amygdala, nucleus accumbens, olfactory tubercle, pontine nuclei, laminae II, III and V of cerebral cortex, and diffusely in corpus striatum and hippocampus. Opioid kappa sites appear to be concentrated in the laminae V and VI of cerebral cortex, caudate-putamen, pyriform cortex, lateral habenulae, bulbus olfactory tubercle, substantia nigra and in the molecular layer of cerebellum. In addition, mu, kappa and delta receptors are found in lamina VI of cerebral cortex, nucleus tractus solitarius, vagus nerve, nucleus ambiguous, substantia gelatinosa of spinal cord, marginal zone and in the laminae I and II of the dorsal horn (Kuhar and Uhl, 1979; Chang, 1984; Akil et al., 1984; Zukin and Zukin, 1981; Paterson et al., 1984). Interestingly, the binding sites in the rabbit cerebellum are almost all of the mu type, while the guinea pig cerebellum seems to contain kappa receptors exclusively (Paterson et al., 1984).

In the brain, these opioid receptors seem to be both pre- and postsynaptically localized in the neuronal membrane but dominantly presynaptically (Kuhar and Uhl, 1979). Biochemical studies have demonstrated that opioids affect the release of neurotransmitters such as acetylcholine, norepinephrine and dopamine, thus suggesting that these receptors on nerve terminals may modulate release of these neurotransmitters (Kuhar and Uhl, 1979; Hagan and Hughes, 1984). Chemical lesion studies with 6-hydroxydopamine in striatum neurons

(Murrin et al., 1980) have suggested that delta receptors may be predominantly localized in presynaptic dopaminergic neurons, while mu receptors seem to be localized in neurons intrinsic to the striatum.

Opioid binding sites have also been detected in the peripheral nerve system, such as in the neurohypophysis (Falke and Martin, 1985) and retinas of rat, cow, toad and snake. The bovine adrenal medulla has mu and delta receptors (Leslie et al., 1980), and kappa receptors are present in placental tissue from both human and rats (Paterson et al., 1984).

In addition, binding studies have demonstrated the presence of specific opioid receptors in the myenteric plexus of the guinea pig ileum but not in the longitudinal muscle itself (Creese and Snyder, 1975). Leslie et al. (1980), reported the presence of two specific binding sites for [^{3}H]-dihydromorphine (mu) and [^{3}H]-D-Ala^2-D- Leu^5-enkephalin (delta) in the mouse vas deferens and guinea pig ileum longitudinal muscle-myenteric plexus membranes, but there are twice as many delta sites as mu sites in the former preparation. So far there is no information on kappa binding studies in these preparations. In the guinea pig ileum myenteric plexus and mouse vas deferens, opioid receptors are located presynaptically within the myenteric plexus and nerve terminals of the vas. Activation of these receptors at low frequency electrical stimulation causes a reduction in release of acethylcholine from cholinergic nerve endings in the ileum (Paton, 1957; Schaumann, 1957; Huidobro-Toro et al., 1981) and a reduction in release of noradrenaline from sympathetic nerve terminals in the vas deferens (Illes, 1982; Wuster et al., 1981a). As a consequence, in both cases the amplitude of excitatory potentials induced by the stimulation of these

nerves is depressed.

Physiological Significance of Opioid Receptors. The physiological significance of the various opioid receptors in the different areas of the central and peripheral nervous system is not yet fully understood. However, it is important to note that these receptors are localized in brain sites related to pain sensation, pain perception and pain modulation, such as the substantia gelatinosa of the spinal cord, the periaqueductal gray, the median raphe, the dorsal medial thalamus and layer IV of the cortex. Creese and Snyder (1975) used binding studies in guinea pig ileum with the myenteric plexus attached and found a high level of correlation ($r = 0.96$, $P < 0.001$), between the ability of opiates to inhibit [^{3}H]-naloxone binding in homogenates of guinea pig intestine and in homogenates of rat brain. In addition, the pharmacologic potency of opiates to depress the electrically-stimulated contractions in the ileum correlates well with the affinity of opiates for receptors in brain homogenates (Creese and Snyder, 1975). It appears that morphine sites in the ileum (mu receptors) resemble those in brain (Chang and Cuatrecasas, 1979) while enkephalin sites in the brain resemble the pharmacological receptor in the mouse vas deferens (Lord et al., 1977; Chang and Cuatrecasas, 1979). Therefore, assays using peripheral nervous tissue such as the guinea pig ileum with the myenteric plexus attached and the mouse vas deferens are very useful preparations to predict the opioid analgesic or antagonistic properties of a compound. In these preparations, the primary criteria for a compound to be acting on opioid receptors is the capacity of naloxone to antagonize the drug-induced inhibition of electrically-stimulated contractions.

Endogenous Opioids

The discovery that the brain possessed stereospecific receptors for morphine led scientists to hypothesize that these opiate receptors interact physiologically with an endogenous substance or substances produced by the body. The first report of an endogenous substance with opioid activity similar to morphine was by Hughes (1975). Hughes used the guinea pig ileum longitudinal muscle and mouse vas deferens preparations and found that extracts from the brains of rabbit, guinea pig, rat and pig were capable of depressing electrically-induced contractions in these tissues. This effect was reversible by naloxone but not by propranolol, phentolamine, hyoscine, hexamethonium, mepyramine and methylsergide, suggesting opioid specificity. The brain extract with its "naloxone-reversible activity" (NRA) was soon partially purified and demonstrated to have a peptide nature. The molecular weight of this substance was estimated to be around 700 or less and its biological activity in the vas was blocked in the presence of aminopeptidases. NRA occurred in brain areas in different proportions, with the highest levels found in the striatum, thalamus, pons and medulla.

Subsequently, Hughes et al. (1975) extended this work by studies done on the amino acid composition of NRA from pig brain and noted that the enkephalins were the endogenous ligand for opioid receptors. The amino acid sequence for the enkephalins was defined as follows: H-Tyr-Gly-Gly-Phe-Met-OH (Methionine enkephalin, Met-ENK) and H-Tyr-Gly-Gly-Phe-Leu-OH (Leucine enkephalin, Leu-ENK). It was also observed that these natural enkephalins were more potent (5-10 times) in vas deferens than in the guinea pig ileum, and that the Met-ENK sequence was present in the sequence (61 to 65) of the long peptide beta-lipotropin,

previously isolated from pituitary glands of sheep, pig and man. At the same time, Goldstein (1976) and Cox et al. (1976) reported that fragments 61-91 as well as 61-65 of beta-lipotropin (beta-LPH) from ovine pituitary demonstrated opioid activity antagonized by naloxone in the guinea pig ileum preparation and that beta-LPH had no effect by itself at concentrations up to 10^{-6}M but in higher concentrations could induce a slow decrease in ileal contractions which could be antagonized by naloxone. This effect was said to be a result of breakdown of beta-LPH to smaller fragments such as beta-LPH (61-91) or beta-LPH (61-65). This same group of investigators also reported that beta-LPH (61-91) was more potent than normorphine in displacing stereospecific binding of [^{3}H]-etorphine in guinea pig brain homogenates, and that beta-LPH (61-65) also was able to inhibit binding but with less potency than normorphine. On the other hand, beta-LPH (1-91) had only a weak effect on binding and beta-MSH [beta-LPH (41-58)] was completely inactive. This beta-LPH (61-91) fragment was later called beta-endorphin (beta-END) (Table III) and was shown to be also present in the brain. Beta-LPH (61-65) structurally corresponded to a peptide reported previously (Hughes et al., 1975), namely Met-ENK. In addition, Li et al. (1977) reported the synthesis and analgesic activity of human beta-endorphin (B_H-End). B_H-END was tested in the mouse hot-plate and rat tail-flick assays and was found to be 17-48 times more potent as an analgesic than morphine when applied centrally and 3.4 times more potent than morphine when injected intravenously. This analgesic effect was antagonized by naloxone. B-END was also isolated from rat pituitary (Rubinstein et al., 1977) and was found to be identical to camel and sheep B-END in its amino acid composition. Interestingly, B_H-END has two amino acid residues

Table III. Amino Acid Sequence of Endogenous Opioids and Their Source.

Endogenous Opioid	Amino Acid Sequence	Source
B_H-Endorphin	Tyr^{61}-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu^{91}-OH	Brain and Pituitary
δ-Endorphin	Tyr^{61}-Gly-Gly-Phe-Met-. . .-Tyr^{87}-OH	Pituitary
γ-Endorphin	Tyr^{61}-Gly-Gly-Phe-Met-. . .-Leu^{77}-OH	Pituitary
α-Endorphin	Tyr^{61}-Gly-Gly-Phe-Met-. . .-Thy^{76}-OH	Pituitary
Dynorphin A	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln^{17}-OH	Porcine Pituitary
Dynorphin B (Rimorphin)	Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr^{13}-OH	Porcine Pituitary
Met-enkephalin	Tyr^{1}-Gly-Gly-Phe-Met^{5}-OH	Brain
Leu-enkephalin	Tyr^{1}-Gly-Gly-Phe-Leu^{5}-OH	Brain
Dermorphin	Tyr^{1}-D-Ala-Phe-Gly-Tyr-Pro-Ser^{7}-NH_2	Frog skin
B-Casomorphin	Tyr^{1}-Pro-Phe-Pro-Gly-Pro-$Ileu^{7}$-OH	Bovine Milk
Kyotorphin	Tyr^{1}-Arg^{2}-OH	Bovine Brain

different from camel beta-endorphin (B_c-END): tyrosine in position 87 instead of histidine and glutamic acid in position 91 instead of glutamine. These structural differences do not change its analgesic activity (Li et al., 1977). Furthermore, other opioid peptides such as alpha-endorphin and Met-ENK were soon isolated from rat pituitaries in addition to a nonpeptide which was identified by its cross reactivity with antiserum to morphine and called "morphine-like compound" (MLC) (Rubinstein et al., 1977). MLC binds to opioid receptors of the mouse neuroblastoma x glioma hybrid cell line (NG 108-15) in a noncooperative fashion, much like morphine, naloxone and Leu-ENK (Blume et al., 1977).

Goldstein et al. (1979, 1981) defined the amino acid sequence of a novel peptide isolated from porcine pituitary a few years earlier (Cox et al., 1975) which demonstrated opioid activity in the guinea pig ileum longitudinal muscle and mouse vas deferens preparations. This peptide was about 700 times more potent than Leu-enkephalin, 200 times more potent than normorphine and 50 times more potent than B_c-endorphin in the guinea pig ileum, but only 3 times more potent than Leu-enkephalin in the vas deferens. This opioid peptide was named dynorphin by Goldstein et al. (1979) because of its extraordinary potency in these isolated tissue preparations. Dynorphin 1-13 is more potent than dynorphin 1-17 (Goldstein et al., 1981). Its amino acid sequence is shown in Table III.

While several other endogenous opioid peptides have been isolated from pituitary and brain of various species (Frederickson, 1984), their discovery is not limited to these tissues, e.g., dermorphin from the skin of the South American frog Phyllomedusa sauvagei (Broccardo et al., 1981) and beta-casomorphin from extracts of lyophilized bovine milk (Casy and Parfitt, 1986). The amino acid sequences of these endogenous opioids

are also shown in Table III.

Precursors. It appears that the endogenous opioid peptides belong to three chemical families, each deriving from an independent precursor. The structural sequences of these precursor proteins have been studied using recombinant DNA techniques (Numa, 1984). The B-LPH/ACTH precursor is a large protein (31 kd), also known as proopiomelanocortin (POMC) and is the source of beta-LPH, corticotropin hormone (ACTH), B-END, γ-LPH, α or B-melanocyte stimulating hormone (alpha or beta-MSH, depending on species) and corticotropin-like intermediate-lobe peptide (CLIP). The isolation and identification of this precursor has been done in both mouse and rat pituitary (Rubinstein et al., 1978; Frederickson, 1984). ACTH and B-LPH are the major products of the anterior pituitary while B-END and α-MSH are predominant in the intermediate lobe.

It was originally thought (Austen et al., 1977) that Met-ENK was formed from B-LPH and B-END. However, the observations that B-END and Met-ENK are differently distributed in brain and pituitary, that they occur in different neuronal systems and that their levels in the brain increase at different rates of development (Frederickson, 1984), led to the assumption that enkephalins have an independent precursor. The enkephalins precursor is a 50,000 dalton protein (not yet been purified to homogeneity) called proenkephalin or preproenkephalin A. This precursor contains four copies of Met-ENK and one copy each of Leu-ENK, Met-ENK-Arg^6-Phe^7 and Met-ENK-Arg^6-Gly^7-Leu^8. It was isolated from chromaffin granules in the adrenal medulla since this organ is a rich source of enkephalins (Frederickson, 1984).

Finally, prodynorphin (preproenkephalin B) is the precursor for

dynorphin and neoendorphin. It was isolated and sequenced from porcine pituitary and produces alpha/beta-neo-endorphin, dynorphin A (Dyn 1-17, Dyn 1-13, Dyn 1-8), dynorphin B (rimorphin) and dynorphin B 1-29 (leumorphin). All these peptides contain a Leu-ENK sequence (Numa, 1984).

Distribution. Endogenous opioid peptides have been detected in many animals (reptiles, lizards, fish, rats, rabbits, guinea pigs, cattle, cats, frogs, mice, monkeys and humans; for a comprehensive review see: Olson et al., 1982).

Radioimmunoassay and immunohistochemical techniques have demonstrated that enkephalins and endorphins are independently distributed throughout the brain (Watson et al., 1978) and peripheral tissues (Hughes, et al. 1977). Enkephalins are distributed very diffusely in the brain and spinal cord while beta-endorphin is present mainly in the pituitary gland and certain regions of hypothalamus.

Enkephalin immunoreactivity is found in high concentrations in the globus pallidus, striatum, hypothalamus, thalamus, brainstem nuclei (nucleus ambiguus, nucleus tractus solitarius, dorsal motor nucleus of the vagus, nucleus parabrachialis dorsalis, etc.) and dorsal horn of the spinal cord (laminae I, II, V, VII and X) (Watson et al., 1977). The intermediate lobe of the rat pituitary also shows enkephalin-immunoreactivity (Rossier and Bloom, 1982). In addition, cell bodies containing both enkephalin-like immunoreactivity and enkephalinergic nerve fibers have been shown to occur dispersed in the brain. Such cell bodies are found in many brain areas, e.g., caudate nucleus, periventricular and supraoptic nuclei of hypothalamus, interpeduncular nucleus, central gray and reticular formation, nucleus tractus

solitarius, olfactory bulb and tubercle, lateral preoptic and suprachiasmatic nucleus, amygdaloid nucleus, hippocampus, neocortex, cingulate cortex, medial nucleus of the optic tract, etc. and laminae II, III and VII of the spinal cord. Enkephalin neurons are found in the hippocampus, parts of the cortex, corpus striatum, parts of the hypothalamus, median eminence, parts of thalamus, reticular formation, periaqueductal gray, locus coerulens, parabrachial nucleus, raphe nuclei, nucleus tractus solitarius, motor nuclei of certain cranial nerves and the substantia gelatinosa of the spinal cord (for comprehensive reviews see: Miller and Cuatrecasas, 1979; Rossier and Bloom, 1982; Kuhan and Uhl, 1979; Frederickson, 1984). In general, the levels of Met-ENK are greater than the levels of Leu-ENK and it has been observed that these two enkephalins occur in separate neuron populations in the globus pallidus, putamen, caudate nucleus and guinea pig ileum (Frederickson, 1984). In general, these neurons are characterized by a short projecting axon, thus suggesting an interneuronal role (Holaday, 1983). Met-ENK has also been detected in the cerebrospinal fluid of normal humans and low levels have been found in cerebral ventricular fluid in intractable pain patients (Akil et al., 1978). Outside the central nervous system, large levels of enkephalins are also found in the adrenal medulla, pancreas and endocrine cells of the grastrointestinal tract (Frederickson, 1984) and hearts of guinea pig, hamster, pig and monkey (Xiang et al., 1984; Lang et al., 1982). Enkephalin-containing neurons are found along the whole length of the intestine of man, mouse, chicken, monkey, pig, rat, cat, rabbit and guinea pig. The highest densities of these fibers are present in the myenteric plexus and circular muscle layers of rat and guinea pig intestine along the entire length of the tract (Miller and Cuatrecasas,

1979; Hughes et al., 1977; Frederickson, 1984). The guinea pig myenteric plexus also contains a high density of cell bodies of enkephalin neurons (Frederickson, 1984). Enkephalin fibers in the intestine are found around the ganglion cells of Meissner's plexus and occasionally seen in the circular muscle. Based on their reactivity to individual antisera, it appears that most enkephalinergic fibers in the ganglion cells are Met-ENK neurons and those in the circular muscle layer are Leu-ENK neurons (Rossier and Bloom, 1982). In addition, the enkephalins in the myenteric plexus are synthesized and stored locally in the intestine and are not derived from the brain or pituitary (Sosa et al., 1977). This suggests that they have a neurotransmitter or neuromodulator role in this tissue.

In general, there is a high correlation between the presence of opioid receptors detected by autoradiography with [^{3}H]-naloxone and the presence of enkephalins studied by immunocytochemistry in brain areas such as the habenula, interpeduncular nuclear complex, nucleus tractus solitarius, parabrachial nucleus, nucleus of the spinal tract of the trigeminal nerve, and the dorsal horn of the spinal cord (Akil et al., 1984).

Beta-endorphin is also distributed inside and outside the central nervous system but, in contrast to enkephalins, its highest concentrations are found in the anterior and intermediate lobes (Rossier and Bloom, 1982; Frederickson, 1984) of the pituitary glands of rat, sheep, pig, cow and man, and in the posterior lobe containing the pars intermedia of bovine pituitary (Ross et al., 1977). In the brain, the highest concentrations of beta-endorphin are found in the hypothalamus (Rossier and Bloom, 1979), in the preoptic and paraventricular nuclei and

in the median emnience. The paraventricular thalamus, periaqueductal gray, medial amygdaloid nucleus, zona compacta of the substantia nigra, zona incerta and locus coeruleus also contain high levels of beta-endorphin (Frederickson, 1984; Adler, 1980). Beta-endorphin immunoreactivity is also present in the limbic system (Olson et al., 1982).

Cell bodies containing beta-endorphin in the brain are particularly localized in the arcuate nucleus of the hypothalamus (Holaday, 1983). These cell bodies send fibers to the median eminence, anterior hypothalamus, preoptic and periventricular nucleus. Additional ascending and long descending fibers project to the periaqueductal gray, reticular formation, stria terminalis (Frederickson, 1984) and to brain stem nuclei such as the nucleus tractus solitarius (Holaday, 1983).

Although the pituitary may be a source for brain beta-endorphin, several studies have demonstrated that pituitary and brain beta-endorphins come from separate origins. While brain beta-endorphin seems to originate from hypothalamic neurons in the arcuate nucleus (Frederickson, 1984), pituitary beta-endorphin is probably synthesized locally. In the pituitary, beta-endorphin immunoreactivity occurs in the same structures also exhibiting ACTH immunoreactivity and it is significant that these two peptides come from the same precursor (Rossier and Bloom, 1982). In addition to the pituitary, beta-endorphin has also been found in the pancreas and in human placenta and semen (Olson et al., 1982).

Like enkephalins and beta-endorphin, dynorphin is also found in the central nervous system and peripheral organs. The highest concentration of dynorphin immunoreactivity is found in the posterior lobe of the

pituitary in rats, pigs, cattle and toads, but not in humans (Goldstein, 1984). Cell bodies containing dynorphin are mainly localized in all hypothalamic nuclei, periaqueductal gray, nucleus tractus solitarius, parabrachial nucleus, and also found in the amygdala and raphe pallidus nucleus. Fibers and terminals of dynorphin neurons are found throughout the brain. Highest densities of fibers are present in the substantia nigra, hippocampal formation, internal capsule and ventral pallidum nucleus. Moderate fiber density occurs in the nucleus tractus solitarius, raphe pallidus nucleus, stria terminalis and accumbens nucleus. A few terminals are also seen in the hypothalamus, cerebral cortex, olfactory tubercle, amygdala, globus pallidus and caudate/ putamen complex. In the spinal cord, dynorphin neurons are present superficially in the marginal zone and deeper in the laminae of the dorsal horn. Low levels of dynorphin have been detected in the dorsal root ganglia of rat and rabbit (for review see: Goldstein, 1984).

In the periphery, dynorphin is present in the whole length of the gastrointestinal tract, pancreas, heart, lungs, skeletal muscle, ovary, liver, kidneys and testis and in bovine adrenal medulla (but not rat). While enkephalins are found in the adrenal medulla mainly associated with the chromaffin cells containing epinephrine granules, dynorphin is associated with those containing norepinephrine. In the guinea pig ileum, dynorphin immunoreactive fibers are present in the ganglia of the myenteric plexus and also in the circular muscle layer. Dynorphin-containing cell bodies are found in the submucous plexus (Goldstein, 1984).

In addition, Met-enkephalin, beta-endorphin, humoral beta-endorphin and dynorphin-immunoreactivity have all been detected in circulating

human blood (Boarder et al., 1982).

Physiological Functions. The endogenous opioid peptides have been implicated in several physiological functions and disease states but, to date, their precise role in regulating bodily function is not completely understood. Their most important physiological function probably involves the states of pain and stress. The control of pain is achieved by the existence of an endogenous pain-suppressing system which arises at the level of the brain stem and descends to the spinal cord (for reviews see: Akil et al., 1984; Fields and Basbaum, 1978; Fields, 1981). Evidence for this endogenous pain-suppressing system came from studies showing that electrical stimulation of specific brain stem nuclei led to a reduction in pain response in animals and man. This analgesia was blocked by naloxone. In addition, several types of stress (cold-swim, food deprivation, intermittent footshock) also induce an analgesia which is partially antagonized by naloxone. Both stimulation- and stress-induced analgesia is accompanied by an increase of endogenous opioids in blood and brain (for review, see: Akil, et al., 1984). Pituitary beta-endorphin and enkephalins seem to be involved in stress-induced analgesia (SIA) since hypophysectomy abolishes SIA while the administration of an enkephalinase inhibitor (thiorphan) potentiates SIA. This potentiation is reversible by naloxone (Akil et al., 1984).

Furthermore, the endogenous opioids seem to be involved in the analgesia induced by administration of placebo (Levine et al., 1978), the analgesia induced by electro-acupuncture (Sjolund et al., 1977) and the analgesia induced by the physiological stress of pregnancy and labor (Gintzler, 1980).

A second physiological function in which the endogenous opioids appear to have important relevance is in the control of cardiovascular function during disease, shock and trauma (for reviews, see: Holaday, 1983; Akil et al., 1984). In several animal models of shock, spinal trauma and other cerebrovascular states, the administration of the opioid antagonists, naloxone or naltrexone, improved survival and circulatory variables (Holaday et al., 1982; Faden et al., 1982) indicating that the endogenous opioids may play a role in certain pathophysiological states.

The endogenous opioid peptides are known to affect the secretion of various pituitary hormones (Ferri et al., 1982) and may be involved in obesity (Atkinson et al., 1985). There are also reports implicating the endogenous opioids in behavioral effects (Beaumont and Hughes, 1979; Olson et al., 1982), taste preference (Lynch and Libby, 1983) and in learning and memory performance (McGaugh et al., 1982). Although very controversial, the endogenous opioids have also been implicated in the pathogenesis of psychoses and abnormal behavior (Olson et al., 1982; Copolov and Helme, 1983; Verebey et al., 1978).

Pharmacology of Opioid Receptors in Guinea Pig Ileum and Mouse Vas Deferens

Originally Paton (1957) established the pharmacologic effects of morphine in the isolated guinea pig ileum. Subsequent studies on morphine and other derivatives by Gyang and Kosterlitz (1966), Kosterlitz and Watt (1968) and Henderson et al. (1972) were conducted using both the guinea pig ileum longitudinal muscle and mouse vas deferens preparations. Following these pioneering studies, the two assays have been widely used by many researchers as pharmacological tools in determining the opioid

receptor selectivity of new compounds. Because these two preparations have been demonstrated to be very sensitive to the effects of opioids, only very small amounts of test compounds are needed for a full evaluation. In addition, both agonist and antagonist activity can be documented using these procedures. Opioid agonist capacity is measured by the ability of a test compound to depress electrically-stimulated contractions and then have the effect reversed by naloxone or some other opioid antagonist. The opioid antagonist activity of a given compound is assayed by its capacity to antagonize the inhibition of contractions induced by prototype opioid agonists (Kosterlitz and Waterfield, 1975).

Opioid agonists have been shown to reduce acetylcholine release from cholinergic nerve endings in the guinea pig ileum (Paton, 1957; Huidobro-Toro et al., 1981) and to inhibit the release of noradrenaline from sympathetic terminals in the mouse vas deferens (Henderson et al., 1972). An opioid antagonist (e.g., naloxone), on the other hand, causes an increase in the output of acetylcholine released during electrical field stimulation of the guinea pig ileum at 0.017 Hz (Waterfield and Kosterlitz, 1975). This action of naloxone in the ileum is presumed to be due to its capacity to antagonize the action of the endogenous enkephalins present in the myenteric plexus-longitudinal muscle.

There are four pharmacological approaches to establish the opioid receptor selectivity of a new agonist in isolated preparations:

(i) A comparison of the concentration of the agonist to depress contractions in the ileum to that required in the mouse vas deferens. For example, the enkephalins are more potent than morphine in the vas deferens and equipotent to morphine in the ileum; this suggests

that enkephalins are interacting at the same receptor site as morphine in the ileum, but at a different receptor in the vas deferens (Lord *et al.*, 1977).

(*ii*) A documentation of the relative sensitivity of the agonist to naloxone antagonism by measuring the apparent dissociation constant (Ke value) for the antagonist in the same tissue. Naloxone has been widely used as the prototype opioid antagonist; however, this antagonist is not specific for any opioid receptor but does have its best affinity at *mu*-sites. Therefore, low Ke values for naloxone antagonism suggest that the agonist is interacting at *mu* receptors, whereas high Ke values indicate interaction with *kappa* or *delta* receptors (Lord *et al.*, 1977; Paterson *et al.*, 1984).

(*iii*) A checking for selective tolerance induced by an agonist to a particular receptor type. For example, preparations made tolerant to morphine demonstrate cross-tolerance to other *mu* agonists but not to *kappa* or *delta* agonists (Schulz *et al.*, 1981).

(*iv*) A demonstration of selective receptor inactivation by using alkylating agents such as B-FNA. This alkylating agent is a selective antagonist of *mu* receptors (Goldstein and James, 1984). In the ileum, B-FNA causes an irreversible blockade of the effects of normorphine and Leu-enkephalin without affecting the effects of dynorphin (Huidobro-Toro *et al.*, 1982; Way, 1984).

The opioid receptor selectivity of an antagonist is, in general, evaluated by its ability to selectively antagonize the effects of mu, kappa and delta agonists in these preparations (Yoshimura et al., 1982c).

The guinea pig ileum contains mainly mu and kappa opioid receptors (Lord et al., 1977; Schulz et al., 1981); however, Su et al. (1981) have also suggested the presence of a sigma or SKF-10,047 receptor in this preparation. On the other hand, the mouse vas deferens contains three types of opioid receptors, namely delta, kappa and mu, since specific agonists acting at each of these receptors are capable of decreasing contractions in this preparation (Miller and Shaw, 1983; Schulz et al., 1984). It is important to note that delta receptors are not present in the ileum and the agonist effects of delta-ligands (enkephalins) in this preparation are probably due to their interaction at mu sites (Lord et al., 1977; Paterson et al., 1984). The selectivity of synthetic opiates and endogenous opioids for each of these receptor sites is very variable. In both preparations (using the criteria of relative potency to depress contraction and relative sensitivity to naloxone antagonism), the most selective mu agonists are: morphine, normorphine, dihydromorphine, sulfentanyl, etorphine and oxymorphone. Their interaction at other opioid receptors is only about 1% of that at mu sites (Chang, 1984; Lord et al., 1977).

Cyclazocine, EKC and other benzomorphans, as well as U-50,488H, are generally used as prototypes for kappa receptors; however, in binding studies (Lahti et al., 1982) it has been demonstrated that 60% of EKC binding to brain tissue is at mu sites and only 40% of total binding is at kappa sites. U-50,488H seems to bind to kappa receptors only with high affinity.

Opioid peptides are agonists at different receptor sites in these two isolated preparations. In addition to their high potency and sensitivity to naloxone antagonism in these tissues (Lord et al., 1977; Yoshimura et al., 1982a; Sanchez-Blazquez et al., 1984a), and the demonstration of cross tolerance (Schulz et al., 1981, 1984) and selective receptor inactivation with B-FNA or B-CNA (Huidobro-Toro et al., 1982; Cox and Chavkin, 1982; Chavkin et al., 1982), studies have suggested that the dynorphin peptides interact with kappa receptors in both preparations. However, the enkephalin peptides and analogues interact at mu sites in the guinea pig ileum and at delta sites in the mouse vas deferens (Lord et al., 1977). On the other hand, beta-endorphin seems to interact with mu receptors in the ileum and at additional sites in the mouse vas deferens (mu and maybe delta?) (Chang, 1984). Additionally, this peptide could also interact with epsilon receptors like those in rat vas deferens. Both Ke values for naloxone antagonism against beta-endorphin in the rat and mouse vas deferens are similar (Goldstein and James, 1984). Beta-endorphin is the only potent opioid peptide active in the rat vas deferens (epsilon) (Chang, 1984) and it is inactive in the rabbit vas deferens (kappa) (Oka et al., 1980b). In binding studies, beta-endorphin is able to interact at both mu and delta receptors (Paterson et al., 1984). Therefore, it appears that beta-endorphin is a potent agonist at mu, delta and epsilon receptors.

It is interesting to note that receptor selectivity for the dynorphin peptides varies according to the length of the chain. Thus, dynorphins 1-11, 1-13 and 1-17 interact with kappa opioid receptors (Goldstein and James, 1984; Huidobro-Toro et al., 1982; Oka et al., 1982; Yoshimura et al., 1982a,b,c) while dynorphin 1-9 exhibits both kappa and

delta agonist activity in these preparations (Wuster et al., 1981a; Yoshimura et al., 1982c). In addition, smaller fragments of dynorphin (dynorphin 1-5 and 1-7) seem to activate preferentially delta receptors (Yoshimura et al., 1982b; Wuster et al., 1981a).

The enkephalins (Met and Leu-enkephalin), DADLE and several Leu-enkephalin analogues, in particular [D-Pen^2,D-Pen^5]enkephalin and [D-Pen^2-L-Pen^5]enkephalin (for review see: Corbett et al., 1984), are very selective delta agonists while N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-Oh (Aib = alpha-aminoisobutyric acid) (ICI-174,864) is the most selective antagonist at delta receptors.

Other antagonists with rather selective potency have also been described. Two benzomorphan antagonists (Win 44,441 and MR 2266) have demonstrated relative affinity for kappa rather than mu sites (Yoshimura et al., 1982c). Recently, Portoghese and Takemori (1985) reported on an even more selective kappa antagonist which is the triethylenedioxy derivative of beta-naltrexamine (TENA).

Apparently, the mu receptors in the guinea pig ileum are pharmacologically different from mu receptors in the mouse vas deferens (Sayre et al., 1983). It was demonstrated that two alkylating antagonists of mu receptors, 17-cyclopropyl-methyl-4,5-alpha-epoxy-3, 14-dihydroxy-6-beta-(iodoacetamido)-morphinan and its fumaramate ester, block morphine effects differently. While the former compound irreversibly antagonizes mu receptors in the mouse vas deferens, no such antagonism exists in the guinea pig ileum. On the other hand, the fumarate ester causes irreversible antagonism of morphine (mu) in the ileum with little or no effect towards morphine in the mouse vas deferens.

Naltrexone is more potent than naloxone against mu agonists prototypes in the guinea pig ileum but not in the mouse vas deferens (Takemori and Portoghese, 1984). This observation also suggests that mu receptors are different in these two preparations.

Purpose of Research

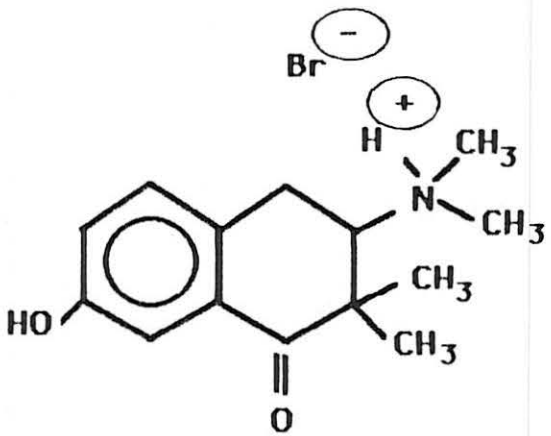
The search for new synthetic compounds with selective agonist and antagonist actions at different opioid receptors has led medicinal chemists all over the world to design hundreds of new structures. Their goal is to find a structure that will produce analgesia without the undesirable side effects and addiction potential of morphine. Since the 2-aminotetralins are structurally similar to morphine, these compounds have the potential for opioid agonist as well as antagonist capacity.

In this research, two new substituted analogues of 3-amino-2,2-dimethyltetralin, namely 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralone HBr (J) and 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralol (MRSAL) (Figure 2) were evaluated for agonist and antagonist activity using the electrically-stimulated guinea pig ileum longitudinal muscle and mouse vas deferens preparations. The chemical synthesis of compound J is fully described in Lippman (1984). There are many reasons for specifically choosing these two tissue preparations:

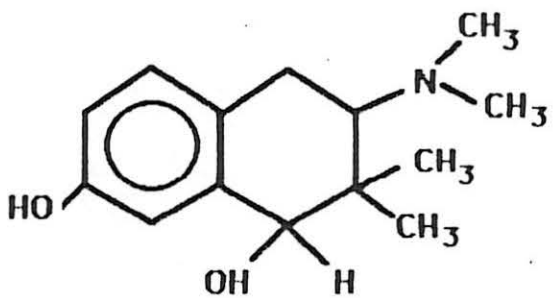
(i) These assays are relatively simple to set up and easy to manipulate pharmacologically.

(ii) These assays are relatively inexpensive and require only small amounts of test drugs.

(iii) Most importantly, these assays are useful in delineating the opioid receptor selectivity of a new



J



MRSAL

Figure 2. Chemical Structures of 3-Dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralone HBr (J) and 3-Dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralol (MRSAL).

compound due to their high sensitivity to opioids and because they contain mu, kappa and delta receptors.

(iv) These isolated tissue assays allow one to predict whether a test compound is an agonist, an antagonist or mixed agonist-antagonist.

(v) These assays make it possible to study the potency, affinity and intrinsic activity of a test compound free from the complex physiological interrelationships observed in whole animal studies.

In this research, we have attempted to define the agonist/antagonist potential of two newly synthesized compounds (J and MRSAL). Agonist potency was compared to that of opioid prototypes such as normorphine and dihydromorphine (mu agonists), ethylketocyclazocine and U-50,488H (kappa agonists), in addition to the peptides B_H-endorphin (mu and delta), dynorphin 1-13 (kappa) and Leu-enkephalin and DADLE (delta and mu). Naloxone was used as the reference opioid antagonist. In addition, the antagonist activity and receptor selectivity of MRSAL was evaluated by its ability to antagonize the effects of the agonist prototypes listed above. It was hoped that the slight difference in chemical structure between J and MRSAL and the dramatic difference in pharmacologic activity might illuminate functional relationships between opioid agonist and antagonist activity and opioid receptor reactivity.

MATERIALS AND METHODS

Drugs and Chemicals

Sodium chloride, biological grade (NaCl), potassium chloride, reagent grade (KCl), calcium chloride dihydrate, reagent grade ($CaCl_2.2H_2O$), potassium phosphate monobasic, reagent grade (KH_2PO_4), sodium bicarbonate, reagent grade ($NaHCO_3$) and dextrose, anhydrous, A.C.S. Certified (d-glucose) were purchased from Fisher Scientific Company (Fair Lawn, NJ). Magnesium sulfate, reagent grade ($MgSO_4.7H_2O$) was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Pyrilamine maleate (Grade 1) and choline chloride (Grade 1) were obtained from Sigma Chemical Company (St. Louis, MO) and L(-) tyrosine, A.C.S. Certified, was purchased from Eastman Organic Chemicals (Rochester, NY). N-Normorphine hydrochloride (NM) and dihydromorphine (DHM) were purchased from Applied Science Laboratories (State College, PA) while ethylketocyclazocine methanesulfonate (EKC) was obtained from Sterling-Winthrop Research Institute (Rensselaer, NY) and naloxone hydrochloride (NX) was purchased from Endo Laboratories (Garden City, NY). [D-Ala^2, D-Leu^5]-enkephalin acetate salt (DADLE) was obtained from Sigma Chemical Company (St. Louis, MO) and the beta-fumaramate methyl ester derivative of naltrexone (beta-FNA) was a gift from Dr. D. Fries of the University of the Pacific (Stockton, CA). Beta$_H$-Endorphin (B_H-END), dynorphin 1-13 (DYN 1-13) leucine-enkephalin (Leu-ENK) and (trans-(dl)-3,4-dichloro-N- methyl-N-[2-(1-pyrrolidinyl) cyclo-hexyl]-benzene-

acetamide) methane sulfonate hydrate (U-50,488H) were generously supplied by Dr. Nancy Lee (University of California, San Francisco, CA). Compounds 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralone HBr (J) and 3-dimethylamino-2,2-dimethyl-7-hydroxy-1-tetralol (MRSAL) were synthesized and donated by D. A. Lippman of Stockton, CA. MRSAL and J are racemic mixtures.

All the reference drugs were dissolved in distilled water, while test compounds J and MRSAL were dissolved in 10% ethanol. The drugs were added to the perfusion bath in volumes ranging from 10-100 µl. Control runs using 10% ethanol alone were performed.

Tissue Preparations

Guinea Pig Ileum Longitudinal Muscle (GPI). Male white guinea pigs (350-550 g), obtained from Simonsen Laboratories (Gilroy, CA), were housed in the animal quarters for one week prior to use and allowed free access to food (Guinea Pig Diet, Simonsen Laboratories) and tap water. Animals were sacrificed by decapitation and the abdomen opened immediately by a midline incision. The ileo-caecal valve was identified and the ileum was removed and quickly placed in Krebs bicarbonate solution at room temperature. The longitudinal muscle myenteric plexus was prepared from a 6-cm segment of ileum as described by Kosterlitz et al. (1970) using the methods of Su et al. (1981). The last 10- to 15-cm portion of the terminal ileum was always discarded. The 6-cm segment of ileum was gently slid onto a pipette of 5 mm diameter and the mesenteric attachment was arranged in a straight line. The strip of longitudinal muscle with its adherent myenteric plexus was then separated from the circular muscle by stroking firmly with a cotton-tipped applicator (soaked in Krebs solution) into the mesenteric attachment and along the

whole segment and circumference of the ileum. By this simple procedure, the longitudinal muscle was completely separated from the circular muscle. The resulting strip consisted of a 6-cm segment of longitudinal muscle with its myenteric plexus firmly attached. The tissue was then transferred to a Petri dish containing Krebs solution and the two edges of the strip were put in juxtaposition and tied together with silk thread (Ethicon, size 4-0) to form a loop 3-cm long. This loop was then mounted between two platinum electrodes in a 5-ml isolated tissue bath (Fig. 3) made to our specifications (Steed Engineering, Palo Alto, CA) containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate buffer solution (pH = 7.1) with the following composition (mM): NaCl 118.40, KCl 4.75, $CaCl_2.2H_2O$ 2.54, $MgSO_4.7H_2O$ 1.20, KH_2PO_4 1.19, $NaHCO_3$ 25.00, d-glucose 11.10, plus choline chloride 20 μM and pyrilamine maleate 0.125 μM. The temperature of the bath solution was kept at 37°C by constant circulation of warm water around the bath using a system of a reservoir and a circulator (Forma-Temp Jr., Forma Scientific Inc.). The muscle strip was connected to a transducer (Grass Force Displacement Transducer Model FT.03) and maintained under a basal tension of 1.0 g. This tension had to be continuously readjusted during the initial 15-20 min period in the bath. The tissue was electrically stimulated (Grass S44 and S88 stimulators) via two platinum ring electrodes made to our specifications (see Fig. 4) with square pulses of 0.25 msec duration at a frequency of 0.1 Hz, using supramaximal voltage (70-80 V). The isometric contractions induced by maximal electric-field stimulation were very regular and consistent and were recorded using a multichannel polygraph recorder (Grass Model 7B Polygraph). After mounting, the preparation was allowed to equilibrate for about 1.3 h with continuous

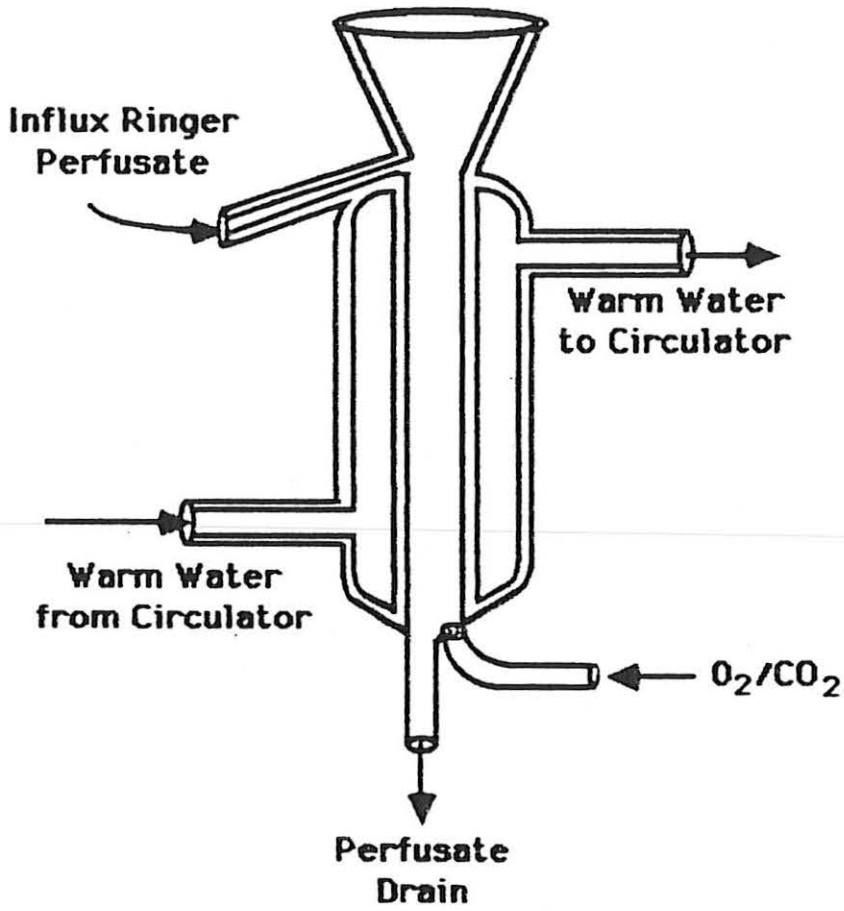


Figure 3. Isolated-Tissue Muscle Bath. Cross-section of a jacketed isolated-tissue bath (5-ml) constructed entirely of glass.

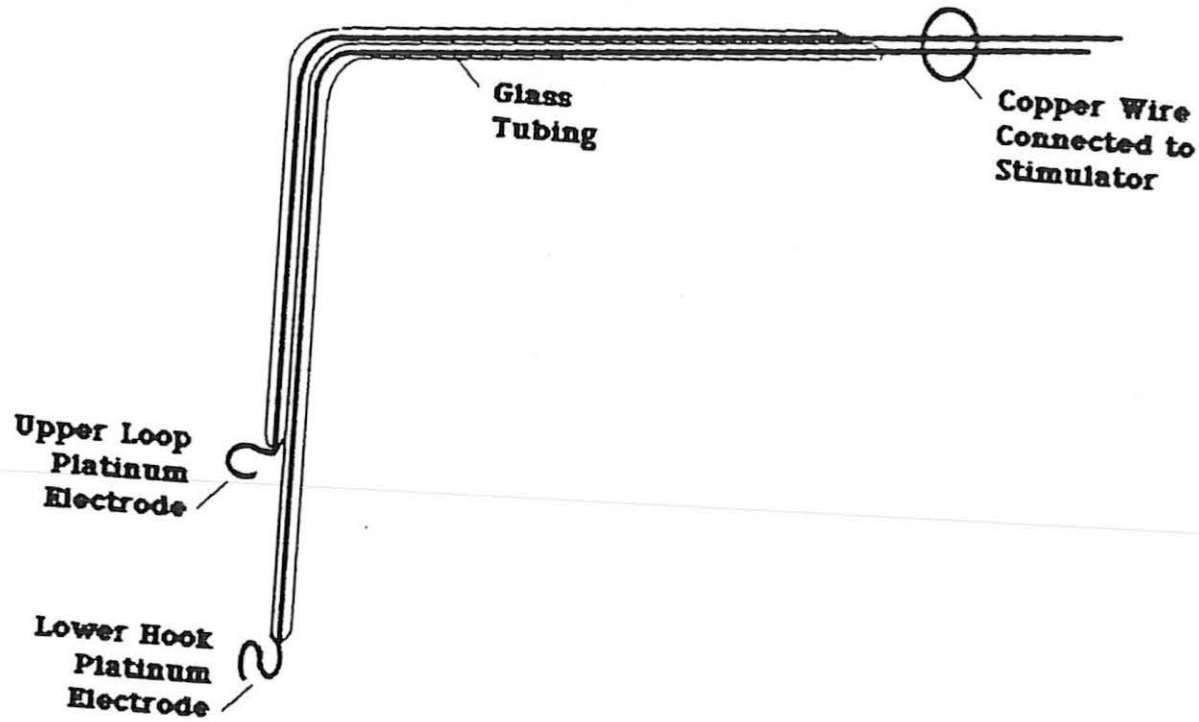


Figure 4. Platinum Wire Electrode for Electrical Stimulation of the Muscle.

electric stimulation and washes every 5-10 min. The tension developed by the longitudinal muscle ranged from 3.0 - 4.0 g.

In general, three to four strips from the same guinea pig were mounted in separate tissue baths in order to assess the variability of tissues from the same animal.

Mouse Vas Deferens (MVD). Male Swiss mice (30-40g) were obtained from Simonsen Laboratories (Gilroy, CA) and housed in the animal quarters for at least 1 week prior to use. The animals were allowed free access to food (Mouse Chow, Simonsen Laboratories) and tap water. After sacrifice by cervical dislocation, the abdomen was opened and the two vasa deferentia were sectioned as closely as possible to the seminal vesicle using the method of Henderson et al. (1972) and then dissected free of connective tissue and excessive fat. The seminal content was expelled by inserting a needle (25 G) into the vas lumen and gently flushing with Krebs solution. Each edge of a vas deferens was tied with silk thread (Ethicon, size 6-0) and the tissue mounted between two rings of platinum electrodes in a 5-ml isolated tissue bath (Figure 3) maintained at 37°C and containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate buffer solution without Mg^{2+}. This perfusion solution (pH = 7.0) had the following composition (mM): NaCl 118.40, KCl 4.75, $CaCl_2.2H_2O$ 2.54, KH_2PO_4 1.19, $NaHCO_3$ 25.00, d-glucose 11.10 and L(-)tyrosine 0.3. One end of the vas was connected to a force displacement transducer and the resting tension of the muscle was set at 0.5 g. This resting tension had to be continually readjusted for the first 15-20 min period in the bath. Washes were made every 5 min. Isometric contractions were recorded polygraphically after being induced

by electric-field stimulation delivered to the tissue via the two electrodes mounted above and below the muscle. The stimulus consisted of square pulses of 2-msec duration at a frequency of 0.2 Hz and with voltage set at 70 V. The vas preparations were allowed to equilibrate for approximately 45 min with frequent washes. Under these conditions, the tension developed by each vas ranged from 0.2-0.5 g.

All the apparatus and instruments (water bath, isolated tissue bath, force displacement transducer, stimulators and polygraph) used in the MVD experiments were identical to those used in the GPI preparations.

Agonist/Antagonist Experiments

Quantification of Opioid Effects. After the preparations (GPI and MVD) had reached equilibrium, the agonist drug was added to the bath and left in contact with the tissue until maximal effect had been reached. Concentration-response curves for all opioid agonist prototypes and for compound J were established using both the cumulative-dose and "single-dose" methods (Kosterlitz and Watt, 1968). The activity of each concentration of an agonist was recorded for 3 min with the synthetic opioids and less than 1-2 min in the case of the opioid peptides.

When the "single-dose" method was used in the GPI, the interval between doses varied from 15-20 min for NM, DHM, B_H-END and Leu-ENK; 15-30 min for J; 25-30 min for DADLE; 25-35 min for DYN 1-13; 30-50 min for EKC; and 80-90 min for U-50,488H. When the cumulative method was used, the interval between doses was 30 min for all agonists except EKC (70 min) and U-50,488H (90-150 min).

When employing the MVD preparation, the interval between doses for all the agonists was 20-30 min.

At least five different concentrations of the agonist were used in the construction of each concentration-response curve. The opioid agonist activity was measured as the capacity of each drug to decrease the electrically stimulated contractions. The concentration of an agonist producing a 50% inhibition of these contractions was termed the IC_{50} and was calculated from the individual concentration-response regression lines.

Determination of pA_2 Values for Opioid Antagonists. In order to assess opioid activity for each agonist, NX was used as a prototype antagonist for the opioid receptors and was either incubated for 3 min before the addition of an agonist or added to the bath at the maximum effect of that agonist. On the other hand, compound MRSAL was incubated for 5 min (MVD) and 5-10 min (GPI) before the addition of an agonist, and this compound was also used to antagonize the decrease in contraction induced by the agonists. When the antagonist was preincubated, muscle contractions were constantly recorded for 3-5 min and after addition of the agonist to the bath, the preparation was either washed out or challenged with the next higher concentration of the agonist being tested. The antagonist potencies of NX and MRSAL against the opioid agonist prototypes and compound J were evaluated by calculation of the pA_2 values for each antagonist. For the calculation of a pA_2 value, concentration-response curves in the absence and presence of the antagonists were generated and the best fitting straight lines were obtained using the least-squares method. The pA_2 values were then obtained mathematically using the equation $A'/A = 1 + B/K_B$ where B = concentration of antagonist used, A = concentration of agonist in the

absence of the antagonist corresponding to observed percent response (obtained from the standard curve) and A' = concentration of agonist in the presence of antagonist. In this instance of $pA_2 = -\log K_B$ (Tallarida and Jacob, 1979).

B-FNA Experiments. These experiments were done only in the GPI preparation. B-FNA (Portoghese et al., 1980; Takemori et al., 1981) was used in order to assess the opioid receptor selectivity of compound J. This experiment was done in three steps: (i) the agonists NM, DHM, J and EKC were each added to the bath at a concentration that would give an approximate 45-60% decrease in contractions and the effects were recorded until maximum effect or for 3 min (time between each dose varied from 30-40 min); (ii) B-FNA (51 nM) was added to the bath, incubated for 45 min while the strip was under continuous stimulation and then the tissue was washed and allowed to recover; (iii) after control contractions were regained, the agonists NM, DHM, J, and EKC were added to the bath at the same concentrations used in step (i). In some experiments, NX and MRSAL were used to antagonize the residual effect of NM, DHM, J and EKC on these B-FNA-treated strips.

Rate-of-Recovery Experiments

Rate-of-recovery experiments were performed for two reasons: first, in preliminary experiments it was observed that the opioid peptides used in this research tended to recover spontaneously after reaching maximal inhibition; second, one way of measuring opioid antagonist activity in the GPI and MVD is by calculating the reversal of contraction induced by the antagonist when added to the bath at the time of the agonist's maximum effect. The rate of recovery for B_H-END,

DYN 1-13 and Leu-ENK was calculated at time intervals of 5, 10, 15 and 20 min. Each rate of recovery was calculated according to the method of Sanchez-Blazquez et al. (1984a) using the following equation:

$$\% \text{ recovery} = \frac{(Tx - Ta)}{(Tb - Ta)} \times 100$$

Where: Tx = amount of muscular contraction (mm) at a specific time interval (5, 10, 15, or 20 min), Ta = amount of contraction (mm) after the agonist had produced its maximum effect and Tb = amount of control muscular contraction (mm) before the agonist was added to the bath. Rate of recovery experiments were performed only in the GPI.

Tolerance Experiments

A few experiments were designed to check if DYN 1-13 could induce acute tolerance in the GPI. In our preliminary experiments, it appeared that with time this peptide lost its capacity to depress the electrically-stimulated contractions.

Three types of experiments were performed. In the first experiment, one concentration-effect curve for DYN 1-13 was done utilizing the "single-dose" method as described by Kosterlitz and Watt (1968). The peptide was added to the bath in increasing concentrations, its effects recorded for 3 min, and the preparation was washed out. Only three concentrations of the peptide were used. The time between doses was 25 min and the total time for completion of the first concentration-response curve was 65 min. A second concentration-effect curve for the peptide was then conducted 25 min later in the same tissue under the same conditions as the first curve. The total time for the second concentration-response curve was 60 min.

In the second experiment, a concentration-effect curve for DYN 1-13 was documented using the cumulative-dose method. In this case, four increasing concentrations of the peptide were used. Each concentration-effect was recorded for 2 min and the preparation was washed out. A second concentration-effect curve was documented in the same preparation 60-80 min later. During this period of time, the preparation was washed every 5-10 min.

In the third experiment, a concentration of DYN 1-13 that would give approximately 50% effect was chosen. This concentration (1.21 nM) was added to the bath and left in contact with the tissue for 10 min in order to check its recovery rate and was repeated at an interval of approximately 30 min. The total time for the experiment was 130 min.

Statistical Analysis

Statistical analysis of data was accomplished with a Hewlett-Packard (model 86B) computer using the Hewlett-Packard HP-83/85 General Statistics Pac. Statistical procedures used were: two-sample *t* test and the paired-sample analysis for calculating basic statistics and linear regression on paired data.

RESULTS

Agonist Potencies in Guinea Pig Ileum Longitudinal Muscle and Mouse Vas Deferens

All compounds tested demonstrated some capacity for depressing the electrically-stimulated contractions of both preparations. The concentration-response agonist curves were mathematically calculated using the linear portion and the least-squares method. Results are shown graphically in Figures 5 and 6. The concentrations (nM) causing a 50% inhibition of maximum response (IC_{50}) as calculated from the regression line equation are shown in Table IV. In the longitudinal-myenteric plexus preparation of the GPI, the most potent compounds were the kappa agonists EKC, DYN 1-13 and U-50,488H (Figure 5) with IC_{50} values (nM) of 1.3, 1.8 and 6.4, respectively (Table IV). The agonist potencies of EKC, DYN 1-13 and U-50,488H as reported by others are summarized in Table V. On the other hand, in the MVD (Figure 6), the delta agonist, DADLE, was the most potent compound tested with an IC_{50} as low as 0.14 nM (Table IV). EKC and DYN 1-13 were less potent in the MVD, but still more potent than mu agonists (NM, DHM) and the delta agonist (Leu-ENK). Leu-ENK was far more potent in the MVD than in the GPI (IC_{50} values (nM) of 19.6 for the MVD versus 408.0 for the GPI). B_H-END, DHM and NM were of intermediate potencies in both preparations (Figures 5 and 6). There appeared to be little difference between the potency of B_H-END in the GPI and in the MVD as was the case for NM and

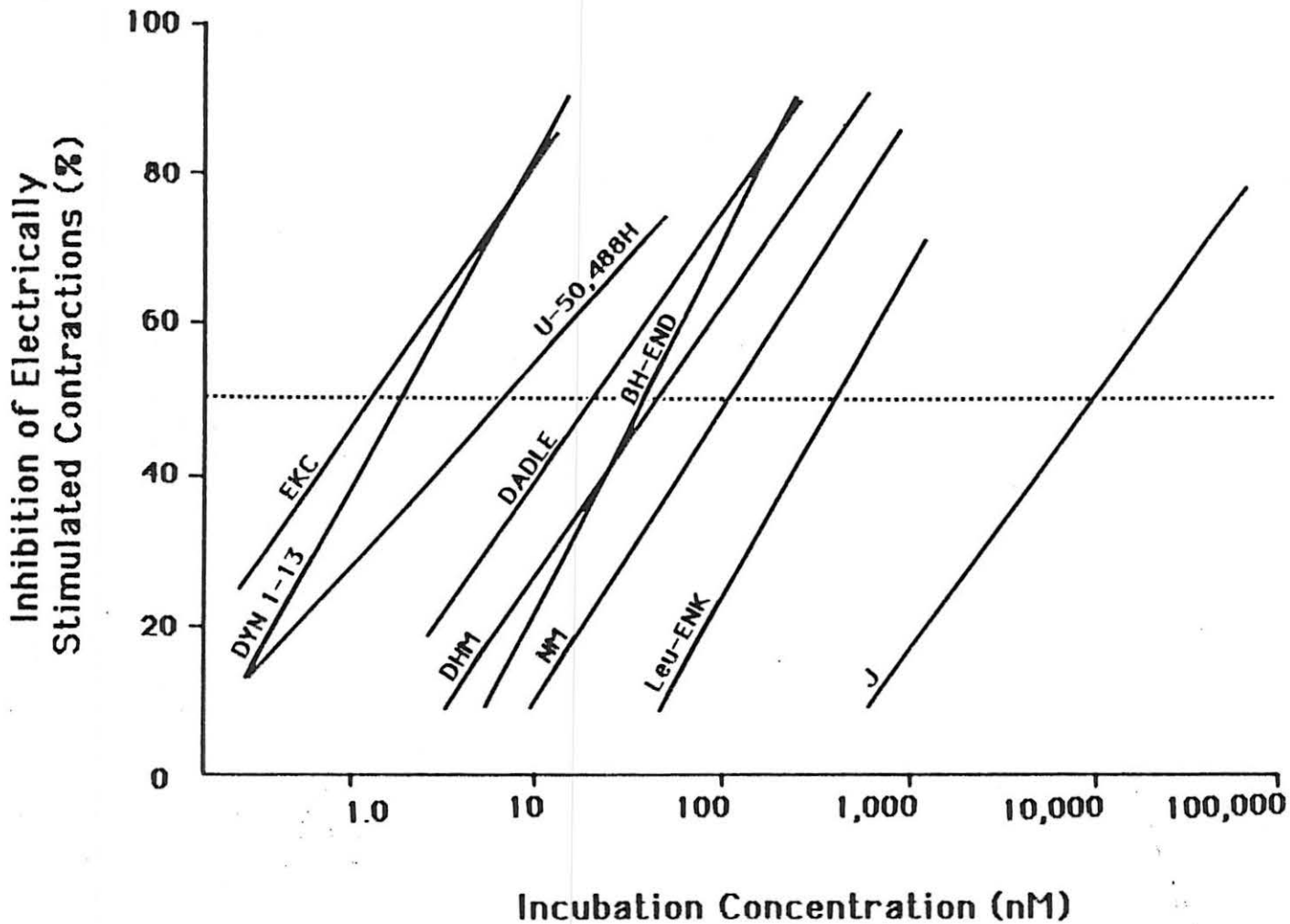


Figure 5. Agonist Capacity of Test and Reference Opioids in the Guinea Pig Ileum Longitudinal Muscle Preparation.

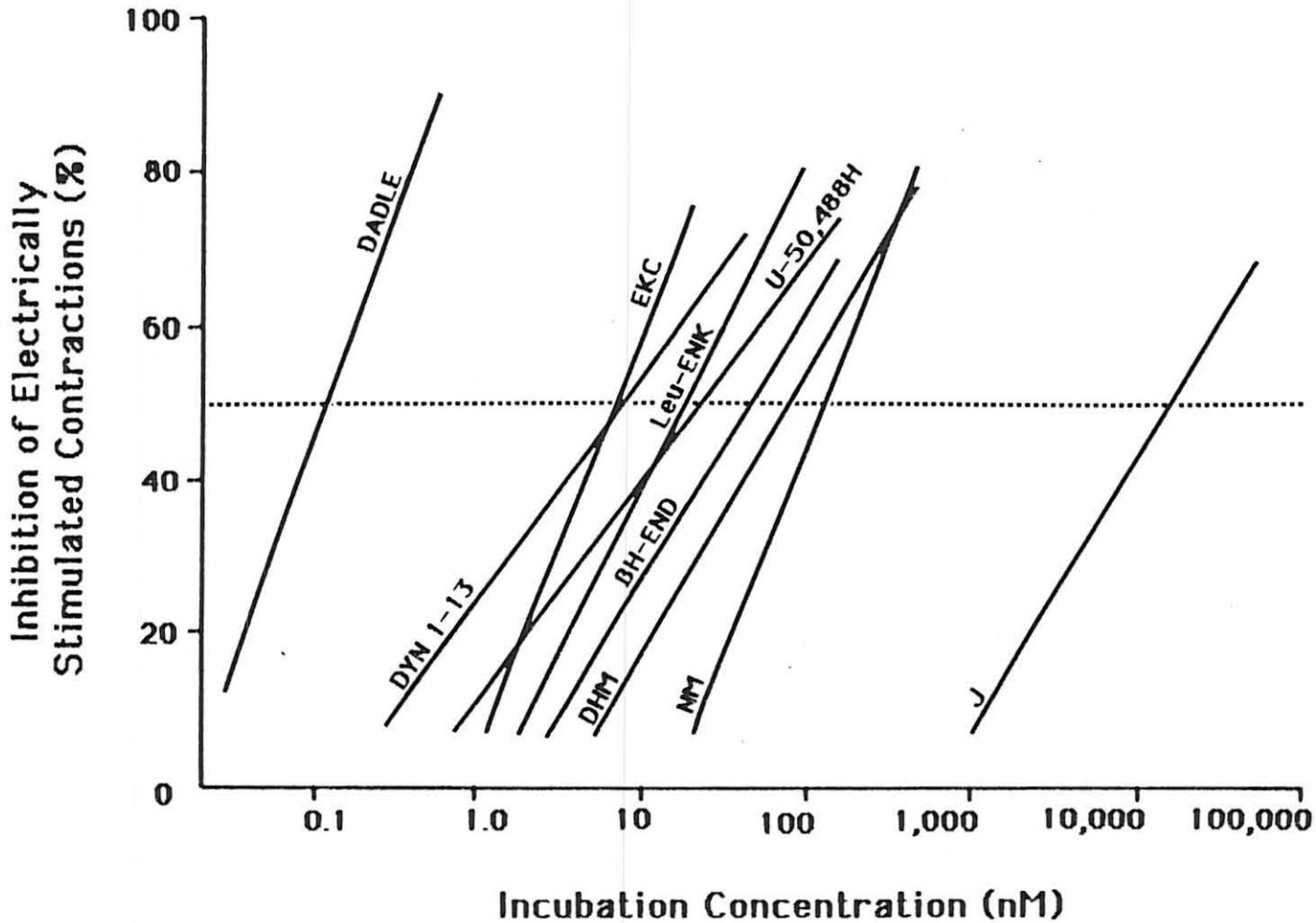


Figure 6: Agonist Capacity of Test and Reference Opioids in the Mouse Vas Deferens Preparation.

Table IV. Agonist Potency of Test and Reference Opioids in the Electrically-Stimulated GPI and MVD.

Opioid Agonist	GPI				MVD			
	N	IC_{50}[a] (95% Confidence Limits)	Slope(b)	r	N	IC_{50}[a] (95% Confidence Limits)	Slope(b)	r
NM	300	110.2 (103.2-117.8)	38.6	0.62	112	132.3 (121.6-143.9)	55.2	0.82
DHM	128	45.1 (40.5-50.1)	35.8	0.72	30	82.7 (67.5-101.2)	37.5	0.72
EKC	135	1.3 (1.2-1.4)	35.5	0.76	37	7.7 (6.8-8.7)	55.8	0.92
U-50,488H	116	6.4 (5.5-7.5)	27.4	0.67	57	24.4 (19.9-30.0)	28.9	0.85
B_H-END	60	37.0 (33.1-41.3)	48.5	0.92	54	48.4 (43.2-54.3)	35.8	0.94
DYN 1-13	196	1.8 (1.7-2.0)	44.4	0.86	70	8.2 (6.9-9.7)	29.8	0.70
Leu-ENK	84	408.0 (366.8-453.8)	43.6	0.86	63	19.6 (17.7-21.8)	42.9	0.91
DADLE	80	19.9 (17.6-22.5)	35.6	0.93	48	0.14 (0.12-0.16)	60.5	0.92
J	174	9,624.9 (8,771.9-10,561.1)	34.1	0.74	70	16,069.4 (13,962.1-18,494.8)	35.9	0.82

[a]IC_{50} = concentration (nM) that would produce 50% inhibition of maximum response as calculated from the respective regression line equations.

Table V. Agonist Potency of EKC, DYN 1-13 and U-50,488H (Kappa Agonists) in GPI as Reported by Other Investigators.

Opioid Agonist	IC_{50} ± S.E. (nM)	Reference
EKC	0.57 ± 0.04	Chavkin et al., 1982
	0.80 ± 0.10	Yoshimura et al., 1982a,b
	0.87 ± 0.07	Yoshimura et al., 1982c
	1.10 ± 0.20	Takemori et al., 1981
	1.21 ± 0.18	Oka et al., 1982
	1.36 ± 0.24	Portoghese and Takemori, 1985
DYN 1-13	0.34 ± 0.04	Chavkin et al., 1982
	0.85 ± 0.09	Yoshimura et al., 1982c
	0.70 ± 0.06	Huidobro-Toro et al., 1981a
	0.70 ± 0.10	Yoshimura et al., 1982b
	2.08 ± 0.26	Oka et al., 1982
	0.79 ± 0.05	Huidobro-Toro et al., 1982
	2.20 ± 0.30	Wuster et al., 1981a
U-50,488H	2.67 ± 0.76	Portoghese and Takemori, 1985
	1.17 ± 0.62	Corbett et al., 1985
	3.60 ± 0.40	Takemori et al., 1986

DHM. Nevertheless, each was more potent in the GPI. The agonist potencies for these opioid agonists in the GPI and MVD as reported by other investigators are summarized in Tables V-IX. In general, the agonist potencies for the reference opioids compounds used in this study are in agreement with those reported by others. However, small differences in IC_{50} values for DYN 1-13, U-50,488H, B_H-END and DADLE in the GPI were noted relative to those reported in the literature. In this study, DYN 1-13 was found to be slightly less potent (IC_{50} = 1.8) than what had been reported by Chavkin et al. (1982), Yoshimura et al. (1982b,c) and Huidobro-Toro et al. (1981a, 1982) (Table V). However, Oka et al. (1982) have reported an IC_{50} value of 2.08 nM which is in close agreement with ours. In addition, the IC_{50} value for U-50,488H (6.4 nM) was found to be slightly higher than that of 2.67 nM reported by Portoghese and Takemori (1985) (Table V). On the other hand, B_H-END and DADLE were found to have lower IC_{50} values (37.0 and 19.9 nM, respectively, Table IV) when compared to those reported by others (Tables VI and VII). In the MVD, there were also small differences in potencies for DADLE while greater differences were observed for EKC and DYN 1-13. DADLE was found to be slightly more potent in this study, an IC_{50} 0.14 nM as compared to the estimates of others of 0.30, 0.50 and 1.92 nM (Table VIII). As shown in Table IX, EKC and DYN 1-13 have been reported to have lower IC_{50} values (0.95 and 3.40 nM) than those found in this study (EKC 7.7 and DYN 1-13 8.2 nM, Table IV). A major discrepancy was observed with the potency of NM in the MVD. This opioid agonist was found to be only slightly less potent in the MVD as compared to the GPI (IC_{50} = 132.3 MVD and 110.2 nM GPI, Table IV). In addition, this agonist was found to have far lower IC_{50} values (132.3 nM) in the

Table VI. Agonist Potency of NM, DHM and B_H-END (Mu Agonists)in GPI as Reported by Other Investigators.

Opioid Agonist	IC_{50} ± S.E. (nM)	Reference
NM	79.0 ± 9.1	Su et al., 1981
	94.0 ± 9.0	Yoshimura et al., 1982b
	108.0 ± 16.0	Yoshimura et al., 1982c
	95.0 ± 9.5	Chavkin et al., 1982
	72.8 ± 17.7	Waterfield et al., 1977
	90.0 ± 4.0	Huidobro-Toro et al., 1982
DHM	62.0	Creese and Snyder, 1975
	38.7 ± 5.5	Leslie et al., 1980
B_H-END	74.0 ± 2.0	Lee et al., 1982
	70.0 ± 2.0	Yoshimura et al., 1982a
	75.0 ± 6.0	Waterfield et al., 1977
	62.0	Paterson et al., 1984
	60.0	Broccardo et al., 1981

Table VII. Agonist Potency of Leu-ENK and DADLE (Delta Agonists) in GPI as Reported by Other Investigators.

Opioid Agonist	IC_{50} ± S.E. (nM)	Reference
Leu-ENK	319.0 (252.7-385.5)[1]	Sanchez-Blazquez et al., 1984a
	613.0 ± 48.0	Yoshimura et al., 1982a
	656.0 ± 90.0	Yoshimura et al., 1982c
	675.0 ± 85.0	Huidobro-Toro et al., 1982
	463.0 ± 59.0	Waterfield et al., 1977
	250.0 ± 20.0	Lee et al., 1982
	220.0 ± 36.0	Chavkin et al., 1982
	303.0 ± 35.0	Fournie-Zaluski et al., 1981
	970.0 ±150.0	Broccardo et al., 1981
	213.0 ± 40.0	Takemori et al., 1986
DADLE	41.2 ± 5.0	Roques et al., 1982
	33.0 ± 3.7	Lee et al., 1982
	47.8 ± 4.4	Kosterlitz et al., 1980
	26.6 ± 7.3	Leslie et al., 1980
	18.2 ± 3.9	Takemori et al., 1986

[1] IC_{50} (95% confidence interval)

Table VIII. Agonist Potency of DADLE and Leu-ENK (Delta Agonists) in the MVD as Reported by Other Investigators.

Opioid Agonist	IC_{50} ± S.E. (nM)	Reference
DADLE	0.85	Sanchez-Blazquez et al., 1983
	0.30 ± 0.05	Lee et al., 1982
	0.50 ± 0.09	Roques et al., 1982
	0.24 ± 0.10	Portoghese and Takemori, 1985
	1.92 ± 0.08	Miller and Shaw, 1983
	0.4 ± 0.05	Wuster et al., 1981a
	0.35	Schulz et al., 1984
	0.54 ± 0.09	Kosterlitz et al., 1980
	0.42 ± 0.04	Leslie et al., 1980
	0.31 ± 0.18	Takemori et al., 1986
Leu-ENK	21.4 ± 3.3	Lord et al., 1977
	7.8 ± 0.8	Waterfield et al., 1977
	7.2 ± 0.5	Lee et al., 1982
	9.8	Sanchez-Blazquez et al., 1983
	8.2 ± 1.6	Fournie-Zaluski et al., 1981
	29.2 ± 3.2	Broccardo et al., 1981

Table IX. Agonist Potency of Several Opioids in the MVD as Reported by Other Investigators.

Opioid Agonist	IC_{50} ± S.E. (nM)	Reference
NM	231.3	Sanchez-Blazquez et al., 1983
	440.0 ± 42.0	Waterfield et al., 1977
	181.0 ± 29.0	Miller and Shaw, 1983
	360.0	Schulz et al., 1984
DHM	156.0 ± 14.0	Leslie et al., 1980
B_H-END	81.7	Sanchez-Blazquez et al., 1983
	80.0 ± 1.2	Lee et al., 1982
	53.0 ± 4.0	Waterfield et al., 1977
	40.0	Paterson et al., 1984
EKC	0.95	Sanchez-Blazquez et al., 1983
	6.06 ± 1.7	Kajiwara et al., 1986
DYN 1-13	3.40	Sanchez-Blazquez et al., 1983
	2.2 ± 0.3	Wuster et al., 1981a
U-50,488H	17.9 ± 7.8	Takemori et al., 1986

MVD than those reported in Table IX: IC_{50} values of 231.3 nM (Sanchez-Blazquez et al., 1983), 440 nM (Waterfield et al., 1977), and 360 nM (Schulz et al., 1984).

Clearly, compound J was the least potent of all the agents tested. In both preparations, J was consistently able to inhibit the electrically-stimulated contractions in a dose-dependent manner, but it had little agonist capacity (Figures 5 and 6). As reported in Table IV, the IC_{50} value for J in the GPI was 9,624.9 and in the MVD 16,069.4 nM. This indicates that J is somewhat more potent in the former preparation. The inhibition of contraction produced by J showed a fast onset of action reaching maximum effect in about 3 min in the GPI and less than 2 min in the MVD (Figures 7 and 8). The maximum agonist response demonstrated by J was about 75% in the GPI and 68% in the MVD.

In general, the opioid peptides demonstrated a greater maximum agonist response (%) than the non-peptides compounds. One exception was seen with Leu-ENK in the GPI which displayed only about 70% maximum agonist activity. In addition, in both preparations, the opioid peptides had much steeper slopes than the other agonists (Table IV and Figures 5 and 6).

Effects of Opioid Antagonists

The effects of NX on the electrically-induced contractions of the GPI and MVD are shown in Table X. In the GPI, this compound demonstrated poor agonist activity, producing a maximum agonist response of about 7% at concentrations of 352 nM, and no agonist response but a 3% increase in muscular contractions at concentrations of 704 nM (Table X). In the MVD, NX also demonstrated poor agonist activity, producing a maximum response of about 9% (Table X).

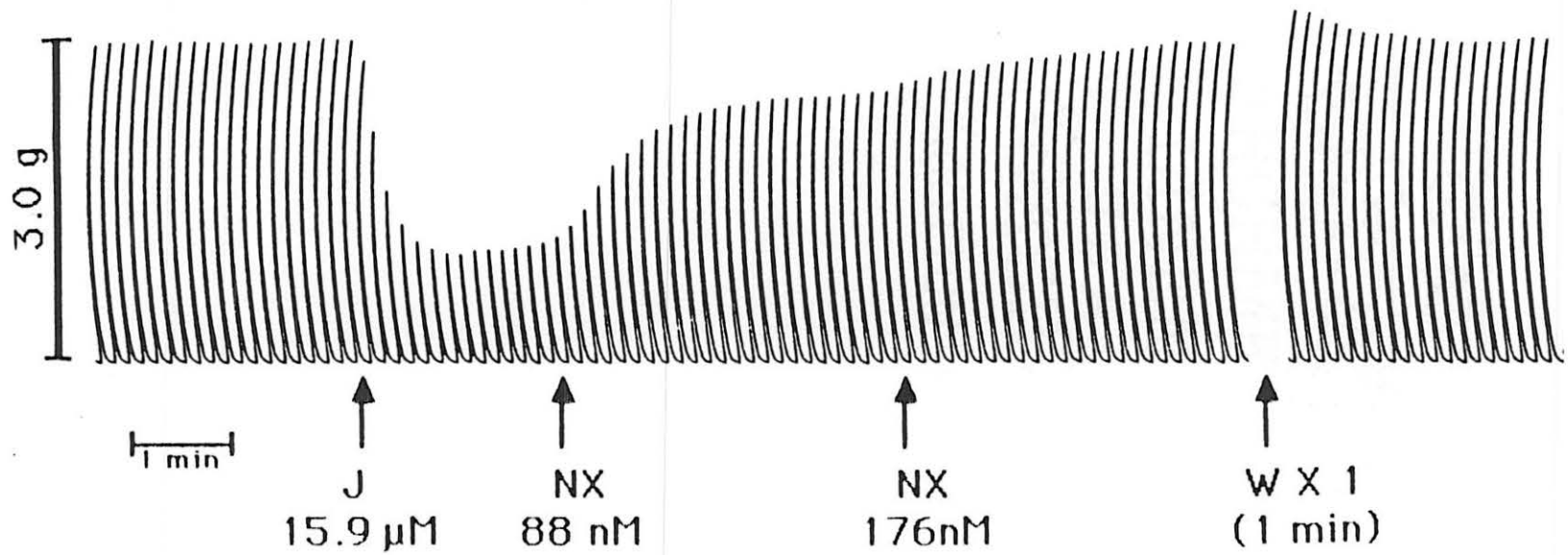


Figure 7. Agonist Activity of J and Its Antagonism by NX in the GPI.

W x 1: The preparation was washed 1 time. Muscular contractions were recorded within 1 min after wash.

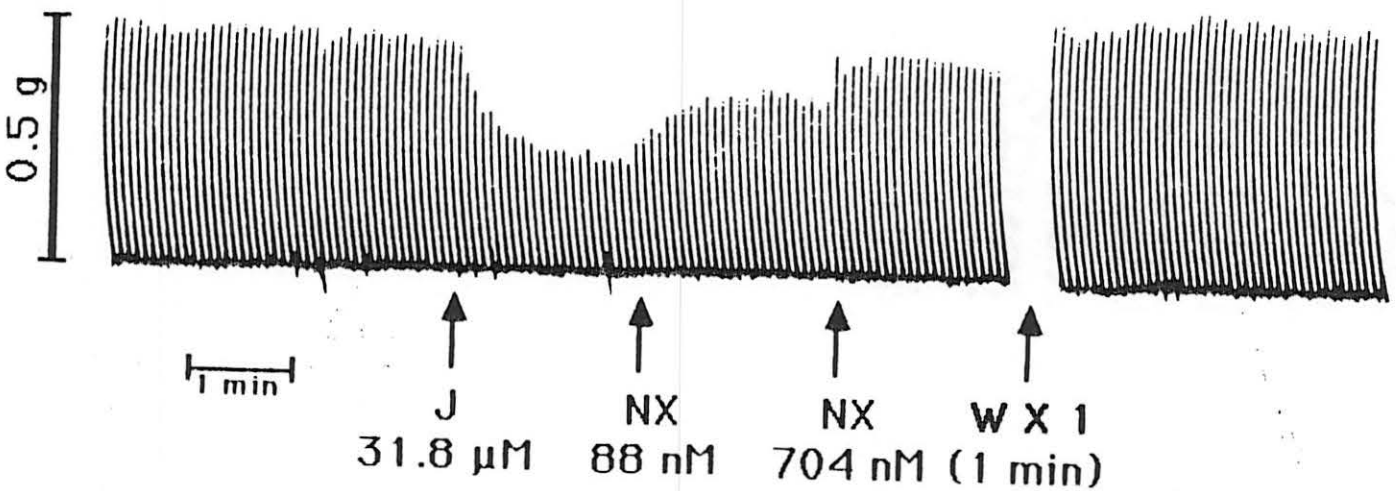


Figure 8. Agonist Activity of J and Its Antagonism by NX in the MVD.

W x 1: The preparation was washed 1 time. Muscular contractions were recorded within 1 min after wash.

Table X. Effects of NX on Electrically-Induced Muscular Contractions of GPI and MVD.

Concentration (nM)	Effect (%)[1] N	GPI[2]	N	MVD[2]
2.8	4	↓2.13 ± 0.31		NT
5.5	4	↓2.67 ± 0.50		NT
11.0	6	↓1.75 ± 0.65		NT
22.0	4	↓3.87 ± 0.86		NT
44.0	7	↓4.65 ± 0.97		NT
88.0	18	↓3.53 ± 0.67	8	↓1.75 ± 1.08
176.0	29	↓4.41 ± 1.36	12	↓7.09 ± 1.53
352.0	13	↓7.45 ± 1.48		NT
704.0	29	↑2.88 ± 1.05	6	↓9.33 ± 1.11

[1]Effects of NX were recorded for 3-5 min.

[2]Results are expressed as Mean ± S.E.

NT = not tested.

On the other hand, compound MRSAL caused an increase in the electrically-stimulated contractions of GPI in concentrations up to 425 μM (Table XI). This increase in muscular contraction was fast in onset--reaching maximum effect in about 3 min and sustained for as long as 15 min. At concentrations ranging from 4.2 to 425 μM, this compound demonstrated a complete lack of agonist activity in the GPI. However, with higher concentrations of 850 μM, (one run only), MRSAL showed some capacity for depressing muscular contractions in this preparation. This decrease in contractions was slow in onset and reached maximum effect about 8 min after introduction to the tissue. The maximum agonist response observed was 76.32% (Table XI). In contrast, in the MVD, MRSAL caused both a decrease and an increase in the muscular contractions at all concentrations tested (Table XI). In general, the increase in contraction in this preparation had a very fast onset of action reaching maximum response in less than 1 min. The decrease in contractions, however, was relatively slow in onset and reached near-maximum response about 5 min after starting incubation. In most cases, a biphasic effect was seen with a very fast increase (30 sec) followed by a slow decrease in contractions. The percent effect reported in Table XI corresponds to the effect seen 5 min after the start of incubation. Testing of the vehicle, 10% ethanol (0.1 ml) produced only small decreases in contractions (about 1%) in both preparations (Table XI).

Opioid Agonist Effect of Compound J

Compound J behaved as an opioid agonist in that it consistently inhibited the muscular contractions of the GPI and MVD in a dose-dependent manner and the effects could be antagonized by NX. J was

Table XI. Effects of MRSAL on Electrically-Induced Muscular Contractions of GPI and MVD.

Concentration (μM)	Effect (%)[1]			
	N	GPI[2]	N	MVD[2]
4.2	30	↑ 0.62 ± 0.40	4	↓18.77 ± 3.47
8.5	21	↑ 2.57 ± 0.32		NT
21.2	91	↑ 4.51 ± 0.27		NT
42.5	19	↑ 3.19 ± 0.54	36	↓ 6.16 ± 2.03
85.0	55	↑ 9.88 ± 1.11	31	↑11.92 ± 4.19
170.0	7	↑11.27 ± 1.19	18	↑12.29 ± 5.14
425.0	3	↑17.14 ± 2.34		NT
850.0	1	↓76.32		NT
Vehicle[3]	10	↓ 1.37 ± 0.53	10	↓ 1.05 ± 0.26

[1]Effects of MRSAL were recorded for 3, 5 and 10 min in GPI and 5 min in MVD.

[2]Results are expressed as Mean ± S. E.

[3]Vehicle - 0.1 ml of 10% ethanol in 5 ml bath = 0.2% ethanol final bath concentration.

NT = not tested.

completely and reversibly blocked by NX at concentrations of 88 and 176 nM. In both preparations, this blockade was effective whether NX was added after the agonist (Figures 7 and 8) or incubated (3 min) before J (Figures 9 and 10). In addition, Figures 9 and 10 show that J was also antagonized by MRSAL in the GPI and MVD.

Antagonist Property of B-FNA in Guinea Pig Ileum Longitudinal Muscle

In order to delineate the opioid receptor selectivity for the agonist effects of J, B-FNA was used as an irreversible antagonist of the mu receptors. In this experiment, NM and DHM were chosen as the prototypes of mu and EKC of kappa opioid receptors. Figure 11 shows the antagonist effect of B-FNA on the effects of NM, DHM, EKC and J on GPI. B-FNA produced an irreversible antagonism on the effects of NM, DHM and J ($P < 0.001$). However, B-FNA (51 nM) also demonstrated significant ($P < 0.01$) antagonism on the effects of EKC.

The antagonism by B-FNA of NM, DHM and J effects was time dependent. B-FNA had to be incubated for 45 min in order to produce significant antagonism. This antagonism was irreversible since NM, DHM and J were unable to cause an effect even after the preparation had been washed thoroughly for more than 20 times and the effect persisted throughout the experiment (450 min). However, B-FNA did not produce 100% antagonism of NM, DHM and J. Therefore, NX and MRSAL were tested to antagonize the residual effects of these agonists on these B-FNA treated preparations. It was found that NX (176 nM) was able to antagonize the residual effect of NM by 100% and J by 98.3 ± 0.8% (mean ± S.E., N = 3). In addition, MRSAL (85 μM) antagonized these agonists by (% mean ± S.E.):

Figure 9. Agonist Capacity of J and Its Antagonism by NX and MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

J (●) 9.6 μM, 34.1 (r = 0.74, N = 174)
J in presence of MRSAL, 8.5 μM (■) 15.2 μM, 36.0 (r = 0.95, N = 35)
J in presence of MRSAL, 21.2 μM (▲) 37.1 μM, 33.5 (r = 0.93, N = 49)
J in presence of MRSAL, 42.5 μM (▼) 52.9 μM, 35.1 (r = 0.93, N = 43)
J in presence of NX, 88 nM (◄) 212.5 μM, 38.8 (r = 0.90, N = 57)

Clear points (□, ▽, ◁) were not used in linear regression.
Please note: Concentrations are in μM.

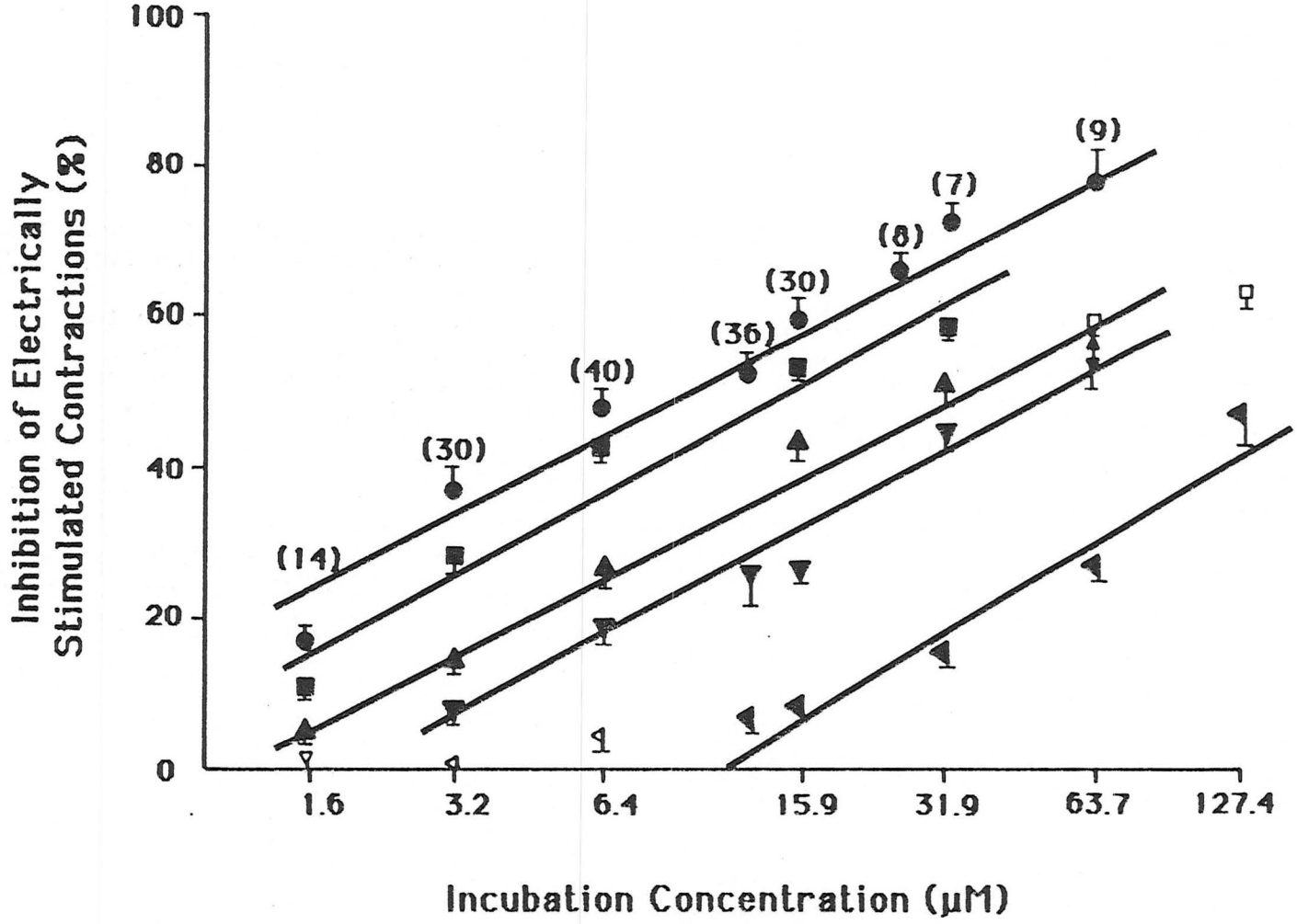

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(14)
(30)
(40)
(36)
(30)
(8)
(7)
(9)
1.6
3.2
6.4
15.9
31.9
63.7
127.4
Incubation Concentration (μM)

Figure 10. Agonist Capacity of J and Its Antagonism by NX and MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

J (●) 16.1 μM, 35.9 (r = 0.82, N = 70)
J in presence of MRSAL, 42.5 μM (▲) 6.4 μM, 30.9 (r = 0.82, N = 31)
J in presence of NX, 176 nM (■) 78.0 μM, 28.1 (r = 0.90, N = 33)

Clear points (○, △) were not used in linear regression.
Please note: Concentrations are in μM.

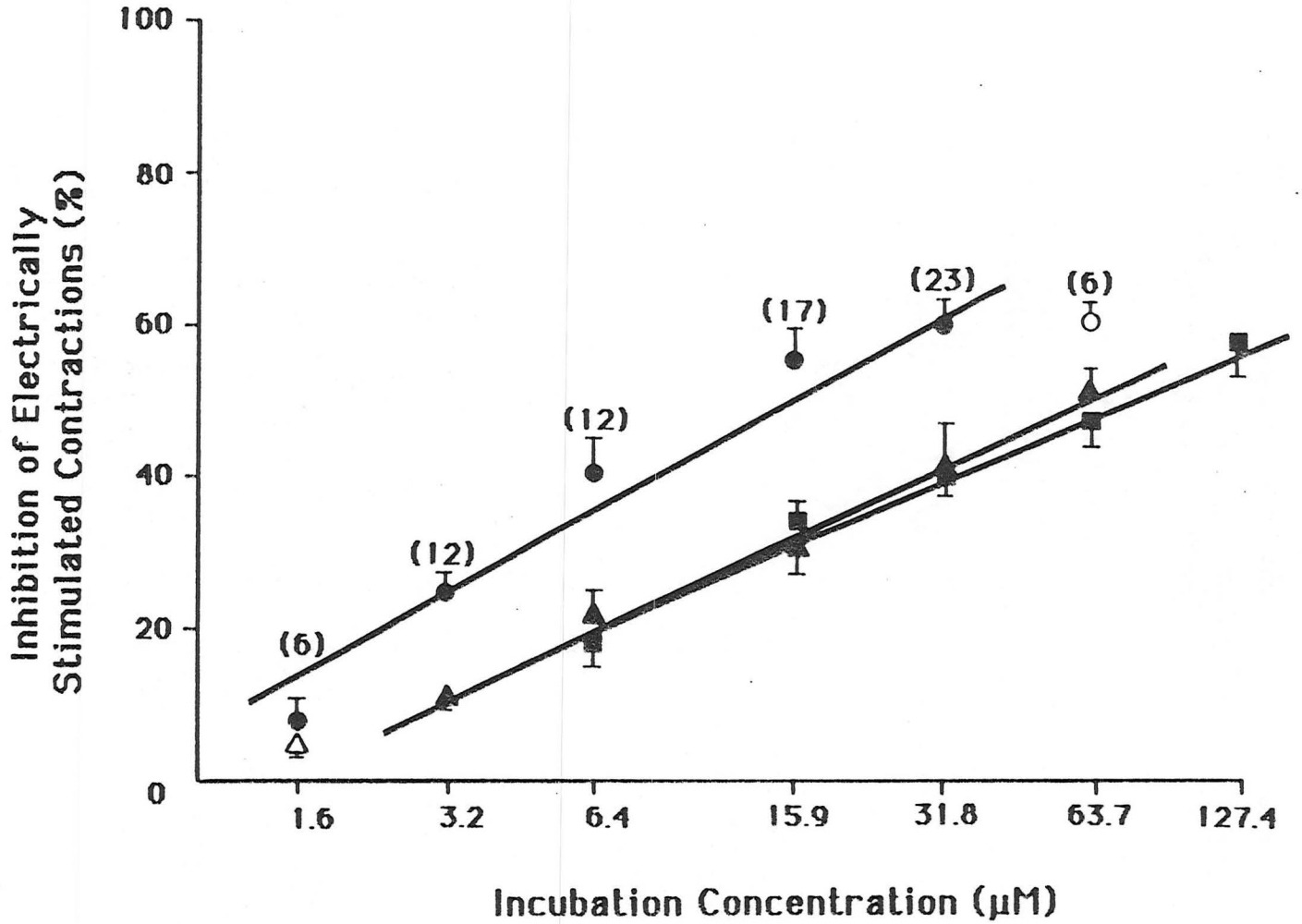
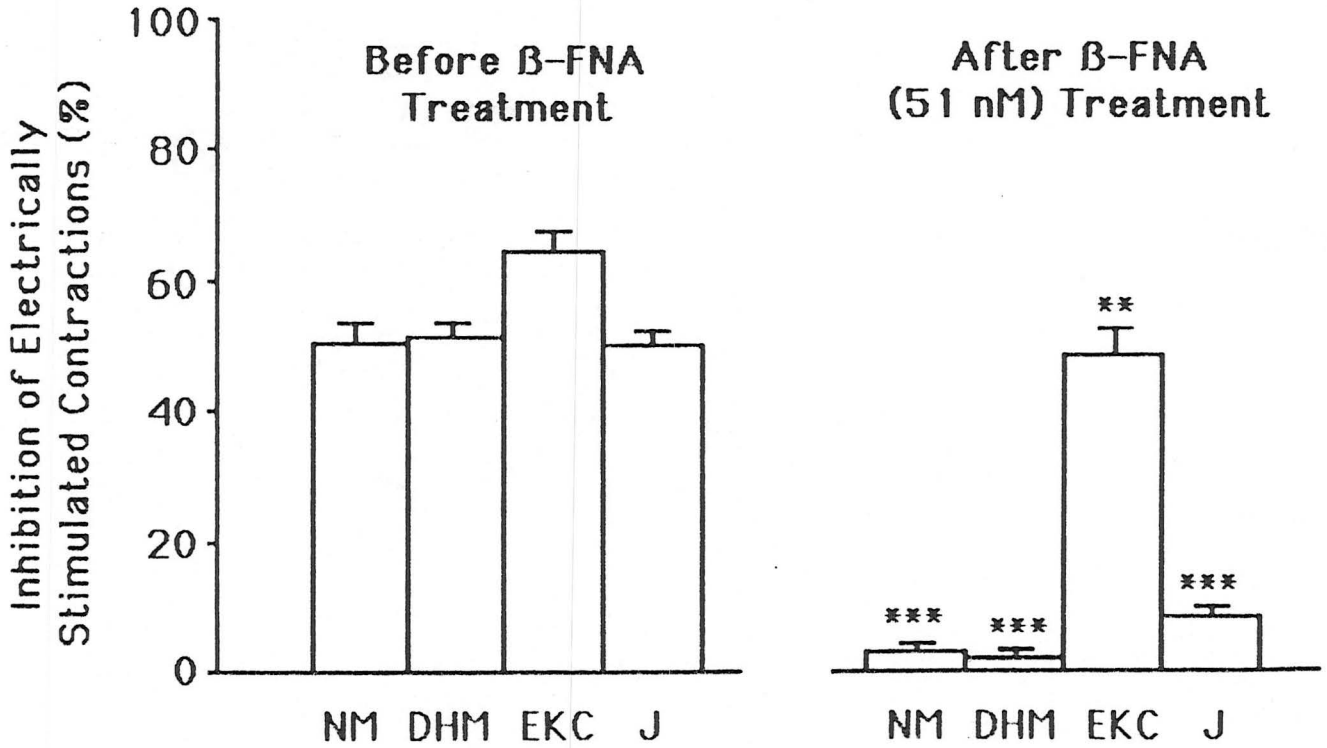

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(6)
(12)
(12)
(17)
(23)
(6)
1.6
3.2
6.4
15.9
31.8
63.7
127.4
Incubation Concentration (μM)

Figure 11. Antagonist Property of B-FNA on the Effects of NM, DHM, EKC and J in the GPI.

Concentrations (nM) used before and after B-FNA treatment were: NM 156.9 (N = 14), DHM 55.7 N = 13), EKC 2.5 (N = 13), J 15,930.0 (N = 10).

Level of significance from control before B-FNA treatment: $\underline{P} < 0.01$ (**), $\underline{P} < 0.001$ (***).


Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
Before ß-FNA Treatment
After ß-FNA (51 nM) Treatment
**
***
***
***
NM DHM EKC J
NM DHM EKC J

NM 99.8 ± 0.21 (N = 6), DHM 99.2 ± 0.4 (N = 3) and J 98.5 ± 0.5 (N = 6). Moreover, MRSAL (170 μM) produced a 56.7 ± 1.0% (N = 3) antagonism of the residual effects of EKC on the B-FNA treated ileum.

B-FNA also possessed agonist activity, producing a decrease in the electrically-induced contractions of 69.1 ± 1.0% (mean ± S.E., N = 50). This effect was fast in onset of action (reaching maximum in less than 2 min) and persisted for the period of incubation (45 min). The agonist effect of B-FNA was reversible; however, it took about 150 min, with washes every 5-10 min, for approximately 80% recovery of control contractions.

Opioid Antagonist Property of MRSAL in Guinea Pig Ileum Longitudinal Muscle and Mouse Vas Deferens

In contrast to compound J, compound MRSAL demonstrated significant opioid antagonist activity in both preparations in a manner similar to that of NX. The antagonism was concentration dependent, reversible and specific. MRSAL was able to antagonize the agonist effects of the opioids used as reference compounds (NM, DHM, EKC, U-50,488H, B_H-END, DYN 1-13, Leu-ENK and DADLE) and also the test opioid agonist, compound J. In addition, MRSAL antagonism was effective whether it was incubated (3, 5 and 10 min) before or added after the agonist. Figures 12 to 27 illustrate MRSAL antagonism when incubated before the opioid agonists in the GPI and MVD. In general, there was a parallel shift of the dose-response curve to the right in a competitive fashion and an increase in IC_{50} values for all agonists tested. However, there was some loss of parallelism when MRSAL was used in higher concentrations in the GPI (Figures 14, 16, 20 and 24). In this preparation, the most effective displacement of the concentration-response curve by MRSAL was

Figure 12. Agonist Capacity of NM and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

NM (●) 110.2 nM, 38.9 (r = 0.62, N = 300)
NM in presence of MRSAL, 8.5 μM (■) 163.3 nM, 54.2 (r = 0.93, N = 30)
NM in presence of MRSAL, 21.2 μM (▲) 377.1 nM, 42.9 (r = 0.86, N = 50)
NM in presence of MRSAL, 85.0 μM (▼) 2,844.5 nM, 34.7 (r = 0.96, N = 55)

Clear points (○, □, ▽) were not used in linear regression.

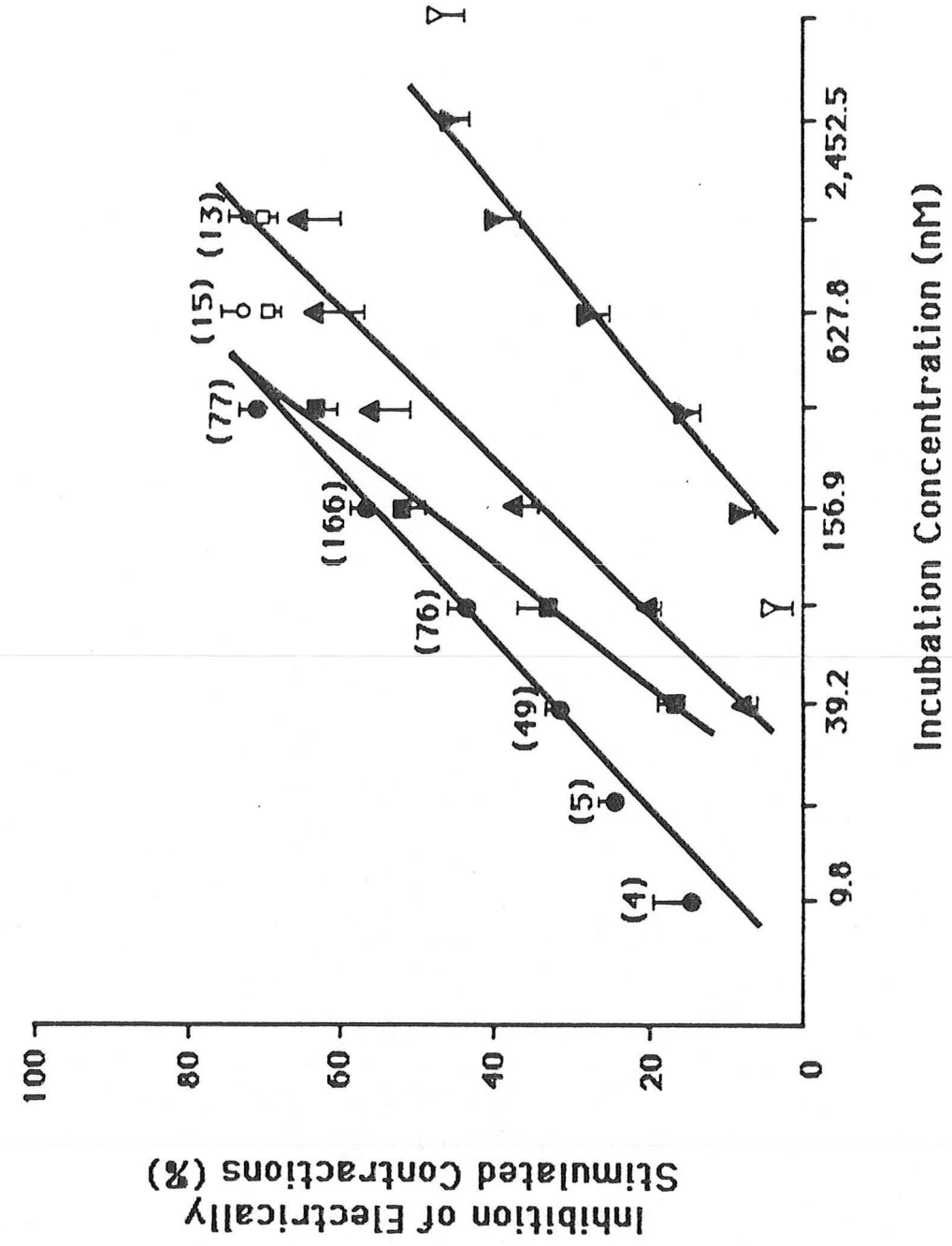

Inhibition of Electrically
Stimulated Contractions (%)
100
80
60
40
20
0
9.8
39.2
156.9
627.8
2,452.5
Incubation Concentration (nM)
(4)
(5)
(49)
(76)
(166)
(77)
(15)
(13)

Figure 13. Agonist Capacity of NM and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

NM (●) 132.3 nM, 55.2 (r = 0.82, N = 112)
NM in presence of MRSAL, 42.5 μM (▲) 305.8 nM, 61.0 (r = 0.93, N = 28)

Clear point (△) was not used in linear regression.

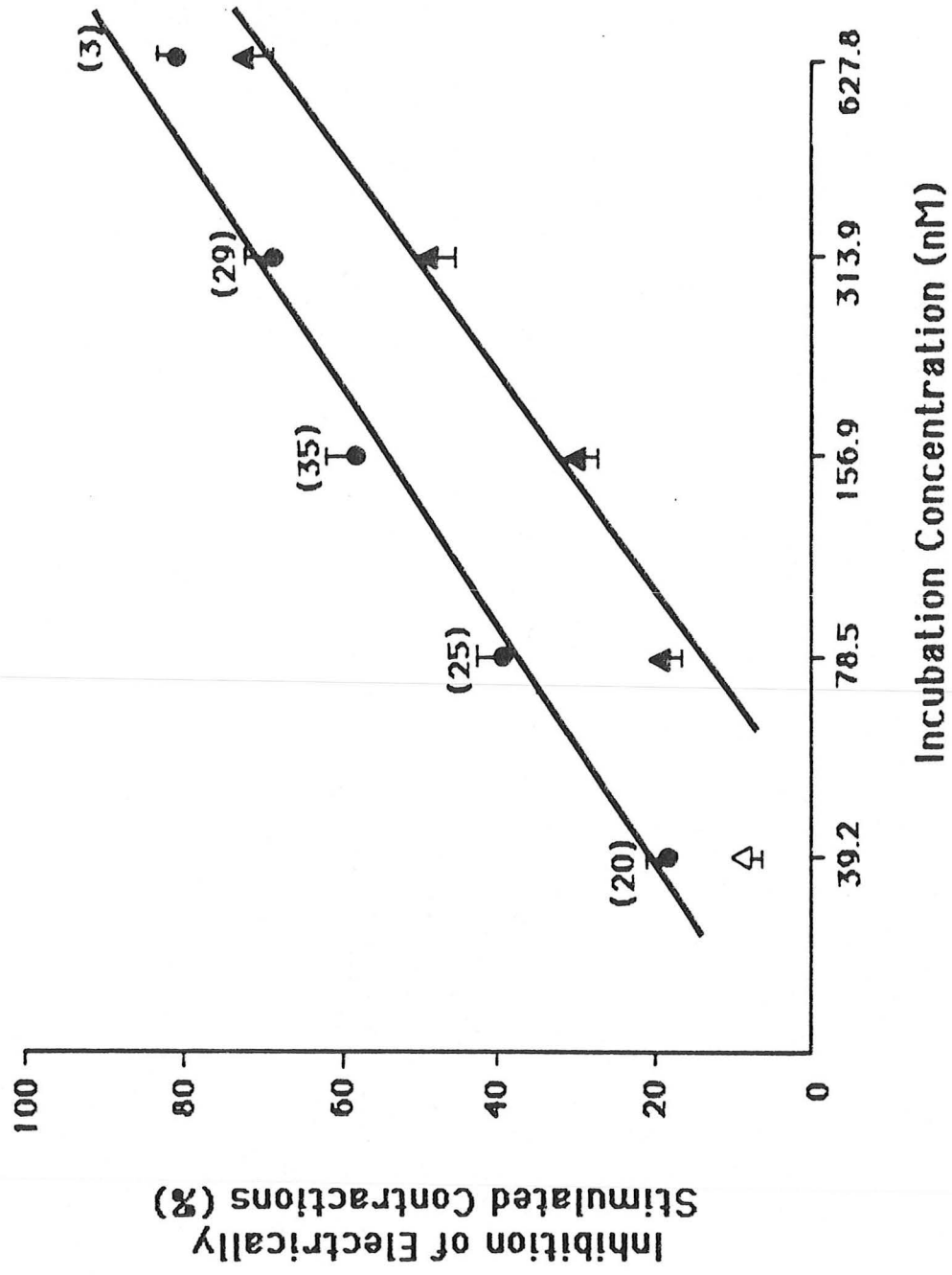

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(3)
(29)
(35)
(25)
(20)
39.2
78.5
156.9
313.9
627.8
Incubation Concentration (nM)

Figure 14. Agonist Capacity of DHM and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DHM (●) 45.1 nM, 35.8 (r = 0.72, N = 128)
DHM in presence of MRSAL, 85 μM (■) 67.0 nM, 31.2 (r = 0.84, N = 35)
DHM in presence of MRSAL, 21.2 μM (▲) 144.2 nM, 41.1 (r = 0.79, N = 43)
DHM in presence of MRSAL, 85.0 μM (▼) 6,478.9 nM, 20.4 (r = 0.86, N = 65)

Clear points (○, □, △) were not used in linear regression.

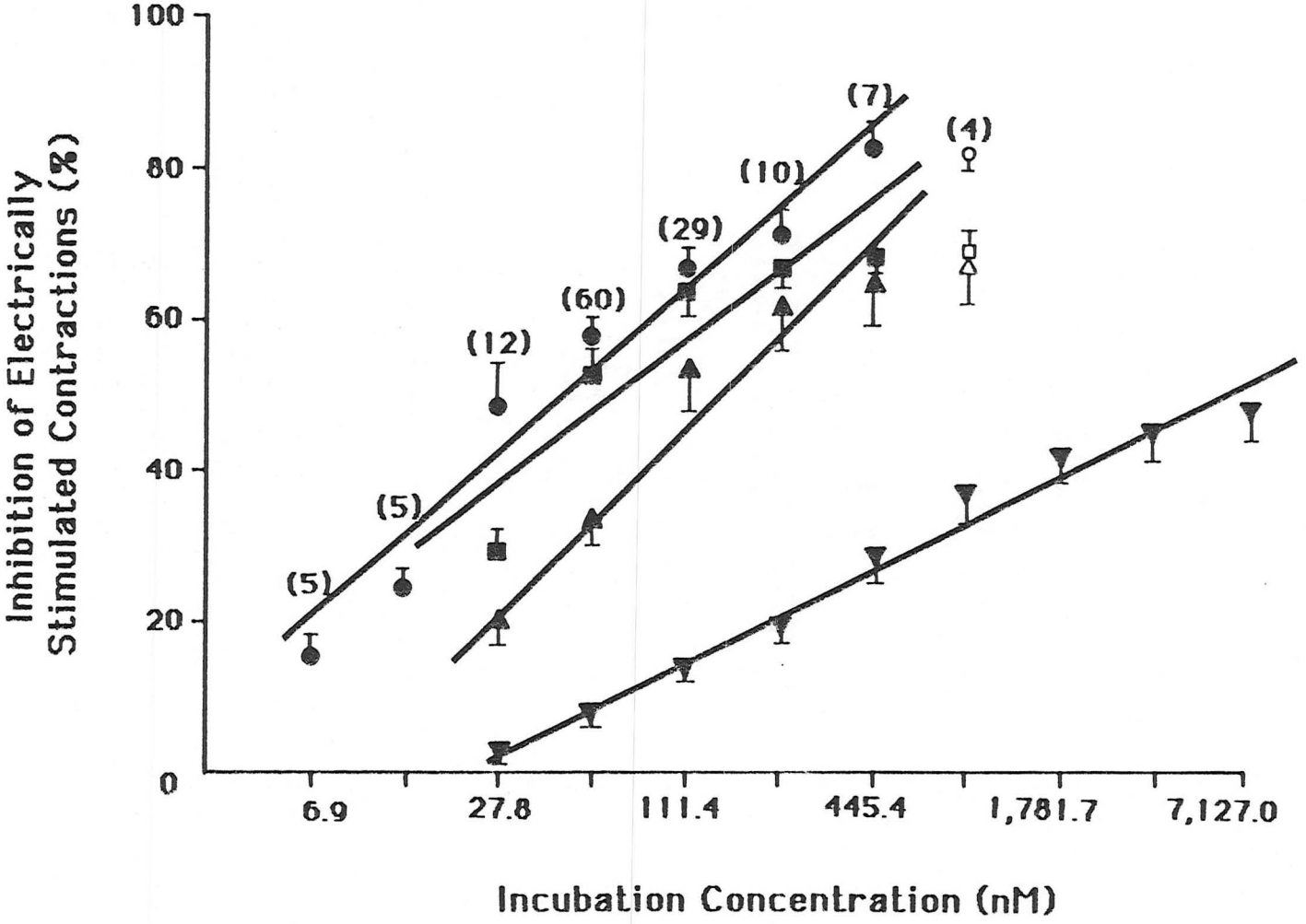

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(5)
(5)
(12)
(60)
(29)
(10)
(7)
(4)
6.9
27.8
111.4
445.4
1,781.7
7,127.0
Incubation Concentration (nM)

Figure 15. Agonist Capacity of DHM and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DHM (●) 82.7 nM, 37.5 (r = 0.72, N = 30)
DHM in presence of MRSAL, 42.5 μM (▲) 346.1 nM, 42.0 (r = 0.88, N = 39)

Clear point (○) was not used in linear regression.

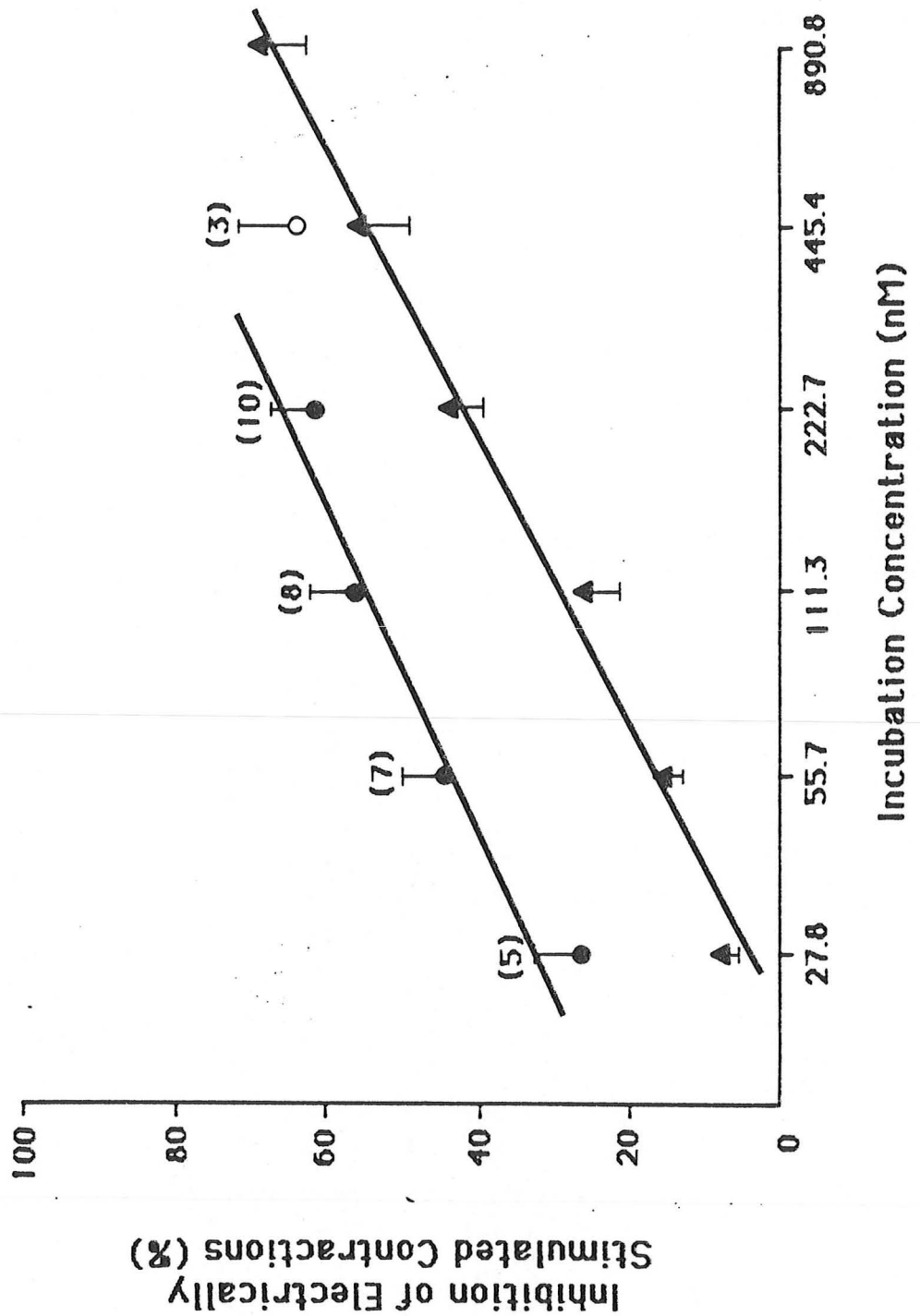

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(5)
(7)
(8)
(10)
(3)
27.8
55.7
111.3
222.7
445.4
890.8
Incubation Concentration (nM)

Figure 16. Agonist Capacity of EKC and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

EKC (●) 1.3 nM, 35.5 (r = 0.76, N = 135)
EKC in presence of MRSAL, 21.2 μM (▲) 2.7 nM, 41.2 (r = 0.82, N = 56)
EKC in presence of MRSAL, 85.0 μM (▼) 19.3 nM, 21.8 (r = 0.90, N = 55)

Clear points (○, △, ▽) were not used in linear regression.

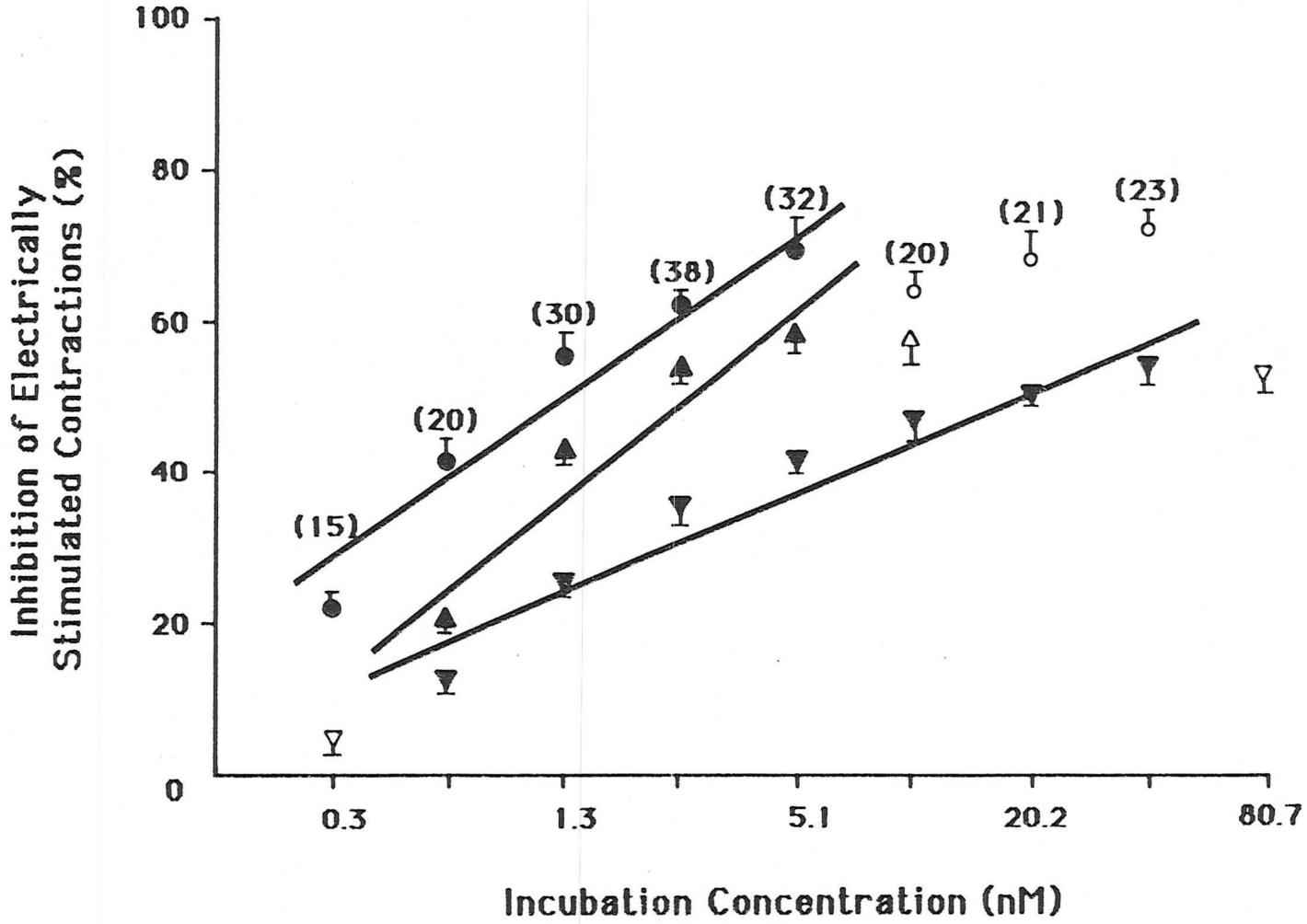

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(15)
(20)
(30)
(38)
(32)
(20)
(21)
(23)
0.3
1.3
5.1
20.2
80.7
Incubation Concentration (nM)

Figure 17. Agonist Capacity of EKC and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

EKC (●) 7.7 nM, 55.8 (r = 0.92, N = 37)
EKC in presence of MRSAL, 85 μM (▲) 17.4 nM, 53.3 (r = 0.93, N = 30)

Clear point (o) was not used in linear regression.

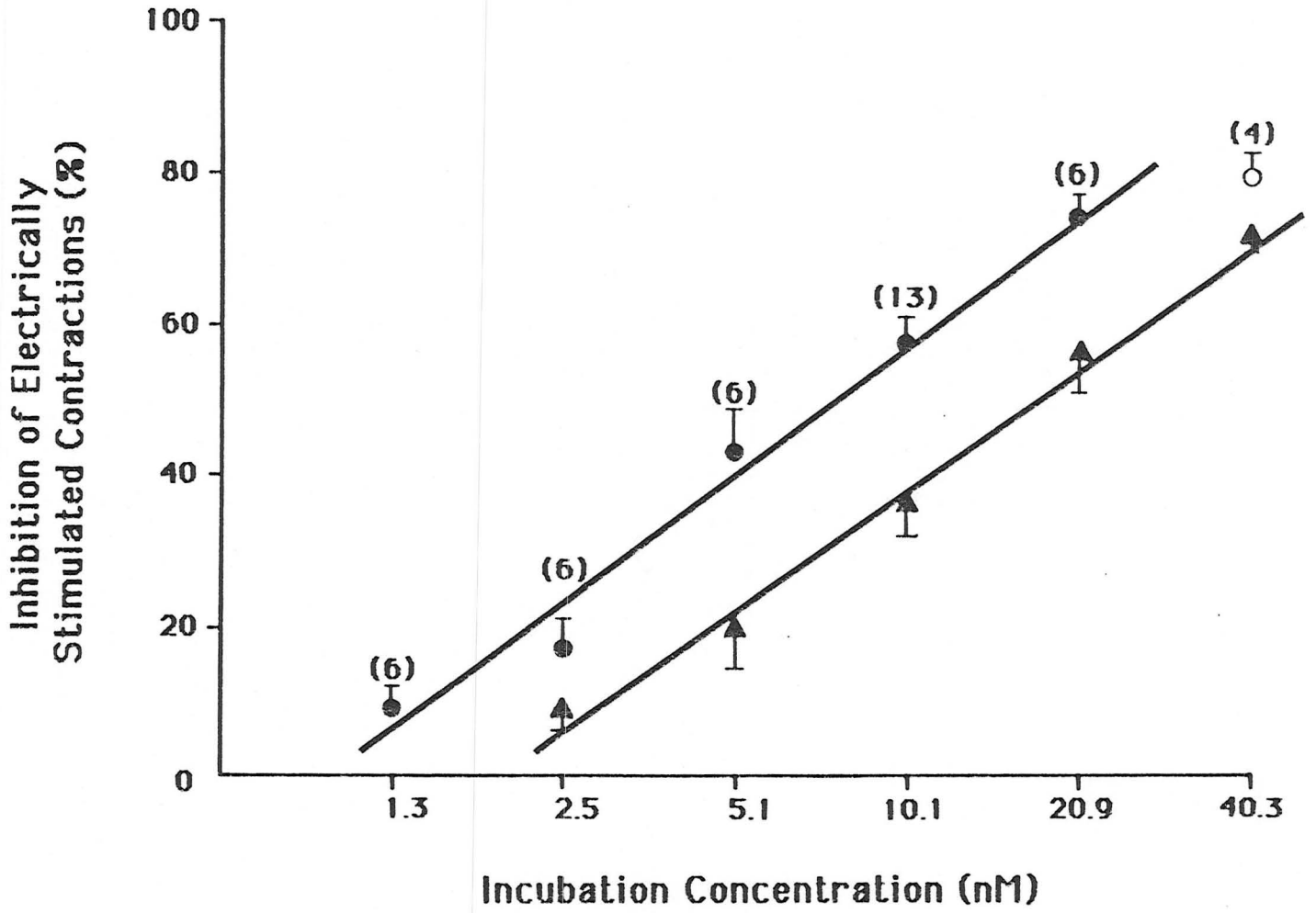

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(6)
(6)
(6)
(13)
(6)
(4)
1.3
2.5
5.1
10.1
20.9
40.3
Incubation Concentration (nM)

Figure 18. Agonist Capacity of U-50,488H and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

U-50,488H (●) 6.4 nM, 27.4 (r = 0.67, N = 116)
U-50,488H in presence of MRSAL, 21.2 μM (▲) 12.4 nM, 41.1 (r = 0.91, N = 70)
U-50,488H in presence of MRSAL, 85.0 μM (▼) 50.3 nM, 29.8 (r = 0.94, N = 42)

Clear points (o, ▽) were not used in linear regression.

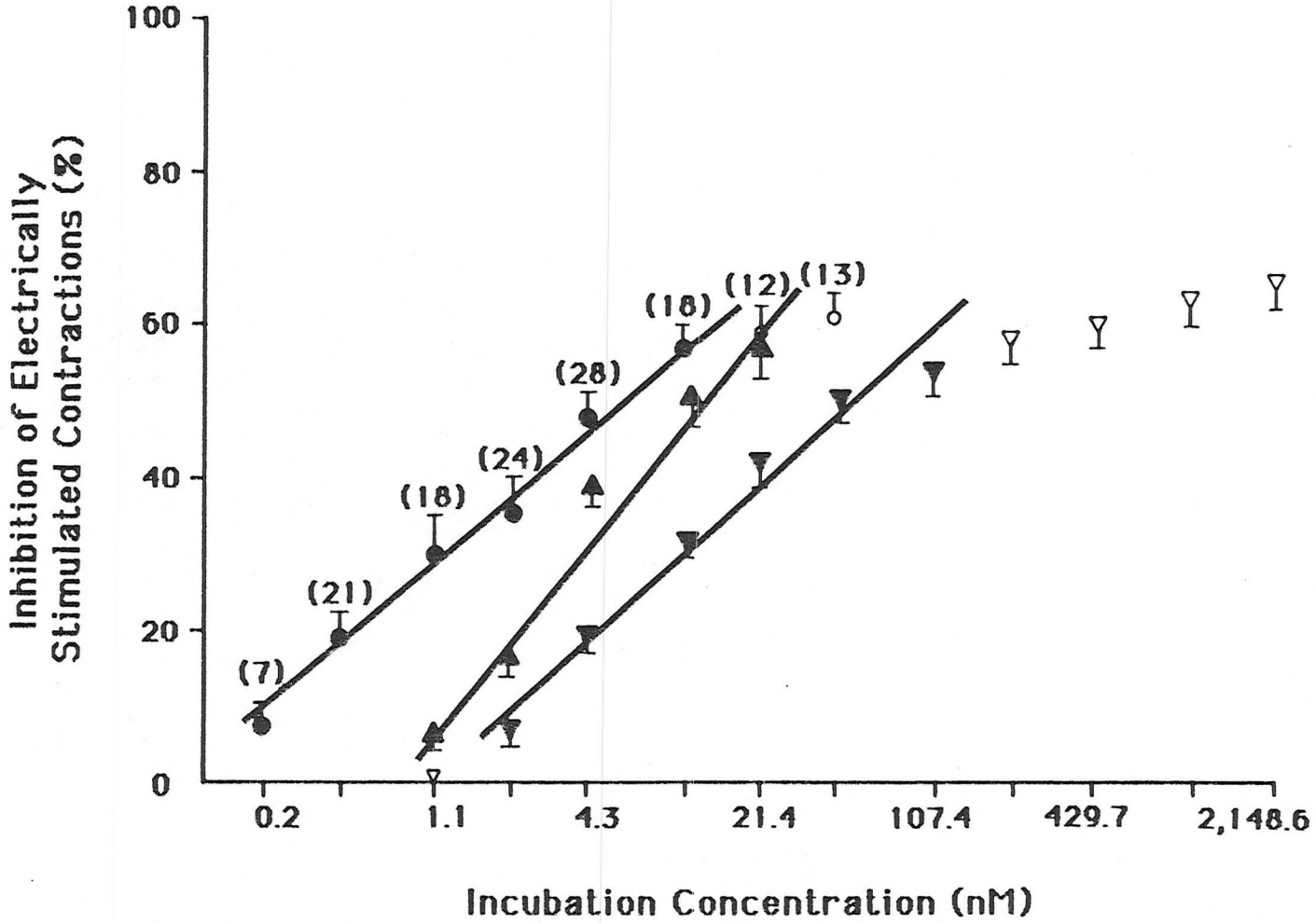

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(7)
(21)
(18)
(24)
(28)
(18)
(12)
(13)
0.2
1.1
4.3
21.4
107.4
429.7
2,148.6
Incubation Concentration (nM)

Figure 19. Agonist Capacity of U-50,488H and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

U-50,488H (●) 24.4 nM, 28.9 (r = 0.85, N = 57)
U-50,488H in presence of MRSAL, 85.0 μM (▲) 162.9 nM, 41.1 (r = 0.82, N = 52)

Clear point (△) was not used in linear regression.

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
1.1
4.3
21.4
107.4
429.0
Incubation Concentration (nM)
(6)
(6)
(6)
(10)
(11)
(10)
(10)
(6)

Figure 20. Agonist Capacity of B_H-END and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

B_H-END (●) 37.0 nM, 48.5 (r = 0.92, N = 60)
B_H-END in presence of MRSAL, 21.2 μM (▲) 228.3 nM, 34.5 (r = 0.87, N = 28)
B_H-END in presence of MRSAL, 42.5 μM (▼) 366.4 nM, 34.4 (r = 0.92, N = 57)

Clear points (△, ▽) were not used in linear regression.

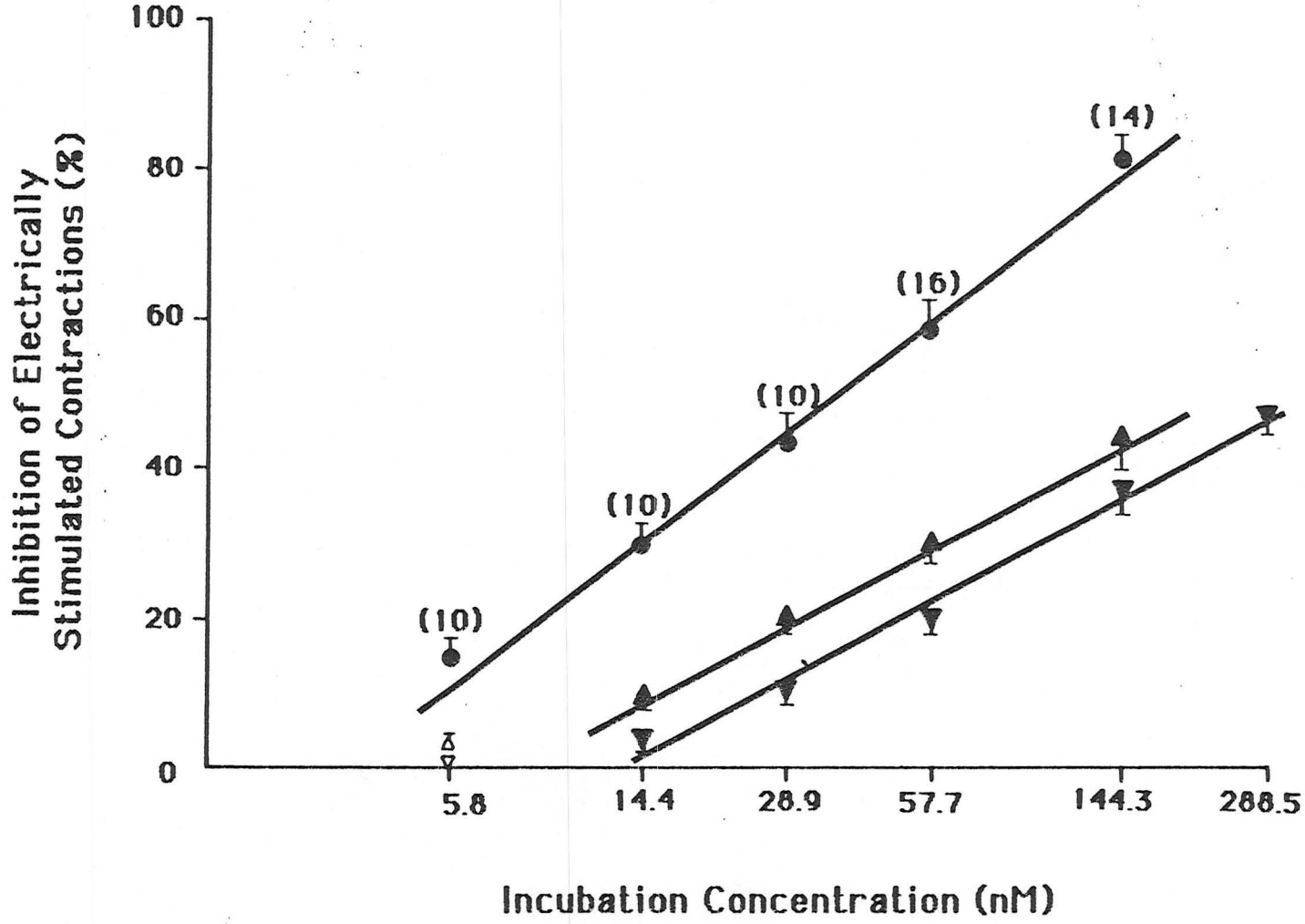

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(14)
(16)
(10)
(10)
(10)
5.8
14.4
28.9
57.7
144.3
288.5
Incubation Concentration (nM)

Figure 21. Agonist Capacity of B_H-END and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

B_H-END (●) 48.4 nM, 35.8 (r = 0.94, N = 54)
B_H-END in presence of MRSAL, 42.5 μM (▲) 76.4 nM, 34.6 (r = 0.91, N = 30)
B_H-END in presence of MRSAL, 85.0 μM (■) 77.3 nM, 43.8 (r = 0.96, N = 42)

Clear point (o) was not used in linear regression.

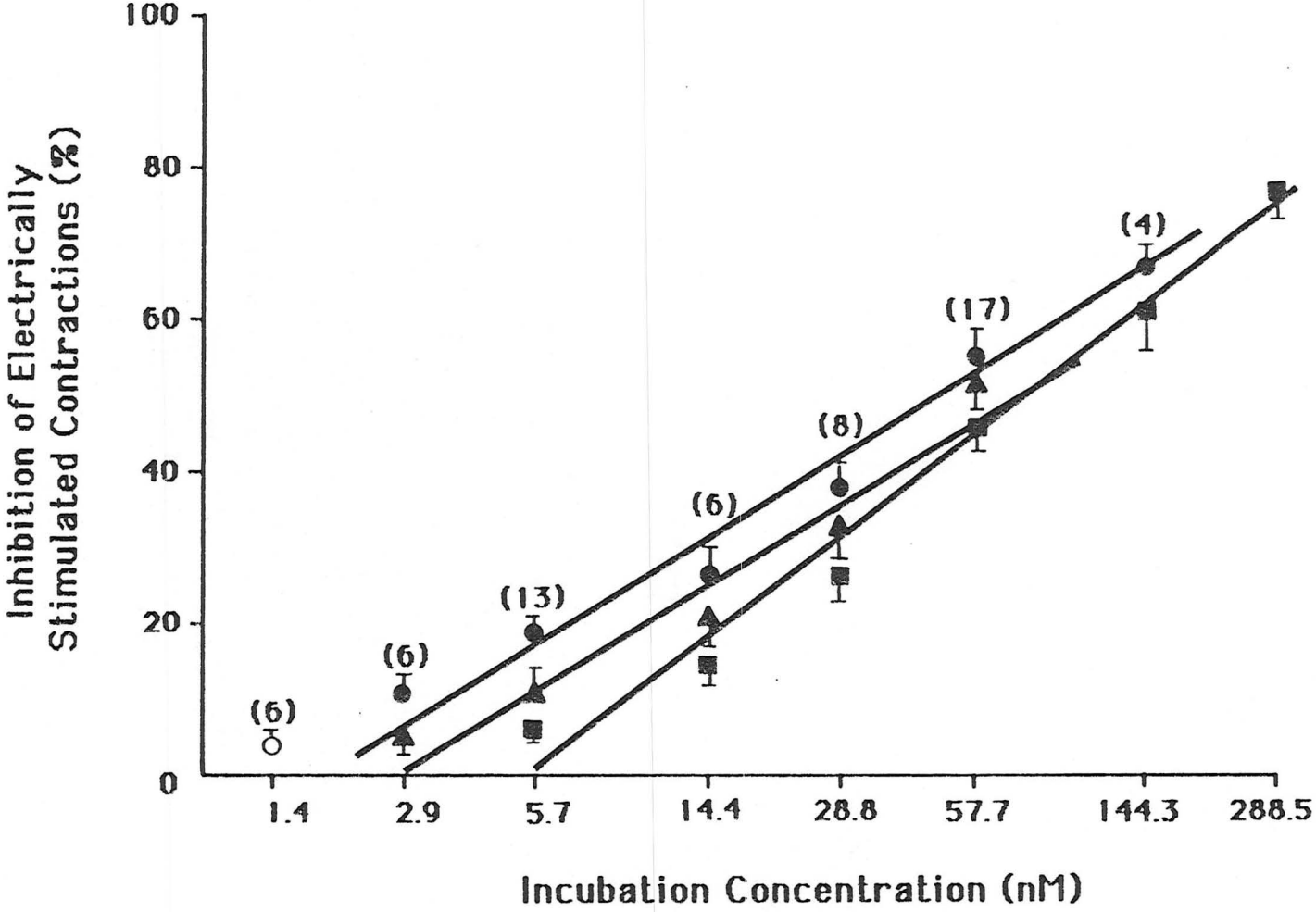

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(6)
(6)
(13)
(6)
(8)
(17)
(4)
1.4
2.9
5.7
14.4
28.8
57.7
144.3
288.5
Incubation Concentration (nM)

Figure 22. Agonist Capacity of DYN 1-13 and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DYN 1-13 (●) 1.8 nM, 44.4 (r = 0.86, N = 196)
DYN 1-13 in presence of MRSAL, 21.2 μM (▲) 3.6 nM, 46.1 (r = 0.91, N = 70)
DYN 1-13 in presence of MRSAL, a85.0 μM (▼) 14.5 nM, 41.8 (r = 0.80, N = 62)

Clear points (o, △, ▽) were not used in linear regression.

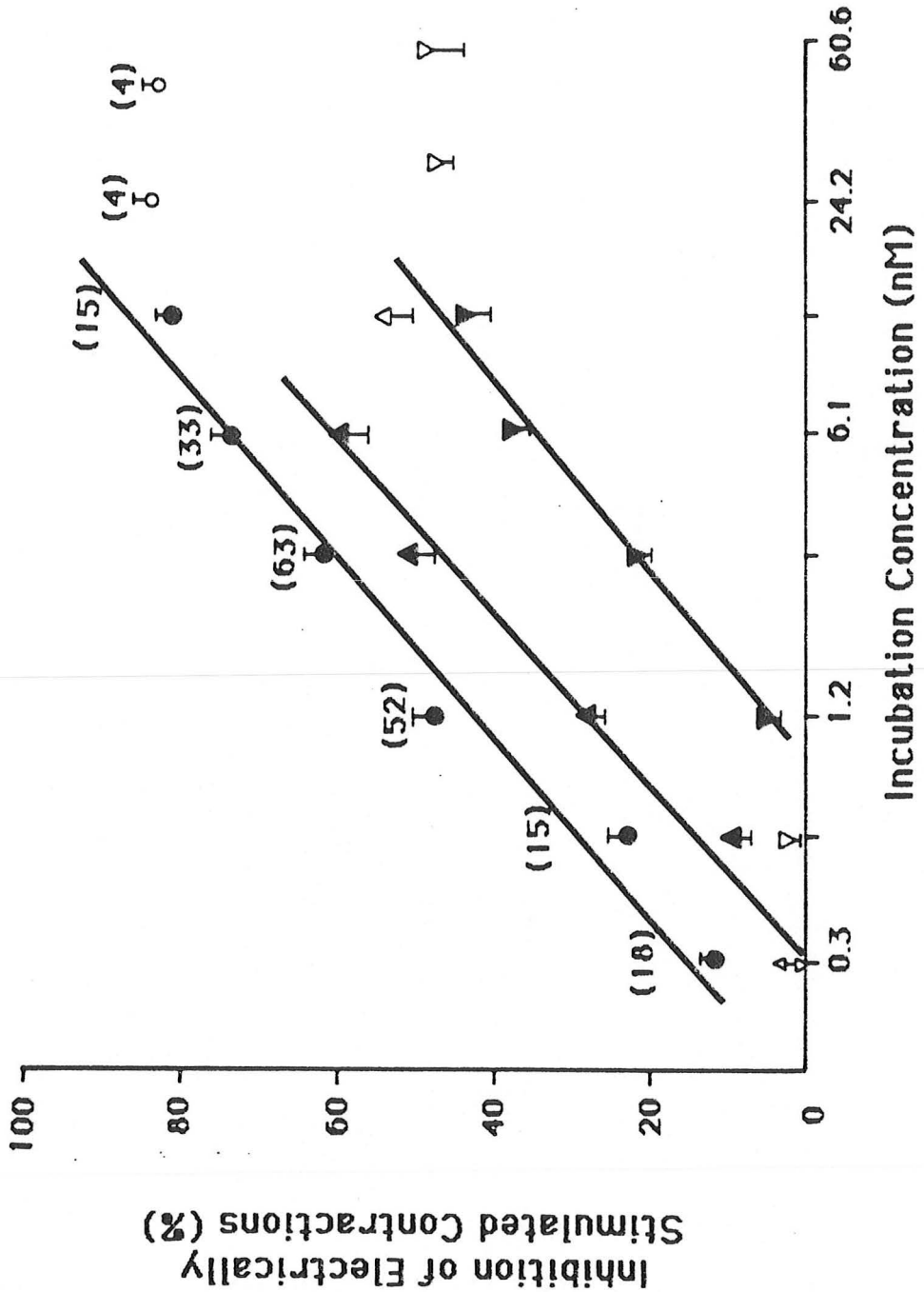

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
0.3
1.2
6.1
24.2
60.6
Incubation Concentration (nM)
(18)
(15)
(52)
(63)
(33)
(15)
(4)
(4)

Figure 23. Agonist Capacity of DYN 1-13 and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DYN 1-13 (●) 8.2 nM, 29.9 (r = 0.70, N = 70)
DYN 1-13 in presence of MRSAL, 42.5 μM (▲) 17.5 nM, 31.2 (r = 0.78, N = 24)
DYN 1-13 in presence of MRSAL, 85.0 μM (■) 63.4 nM, 31.9 (r = 0.93, N = 36)

Clear points (△, □) were not used in linear regression.

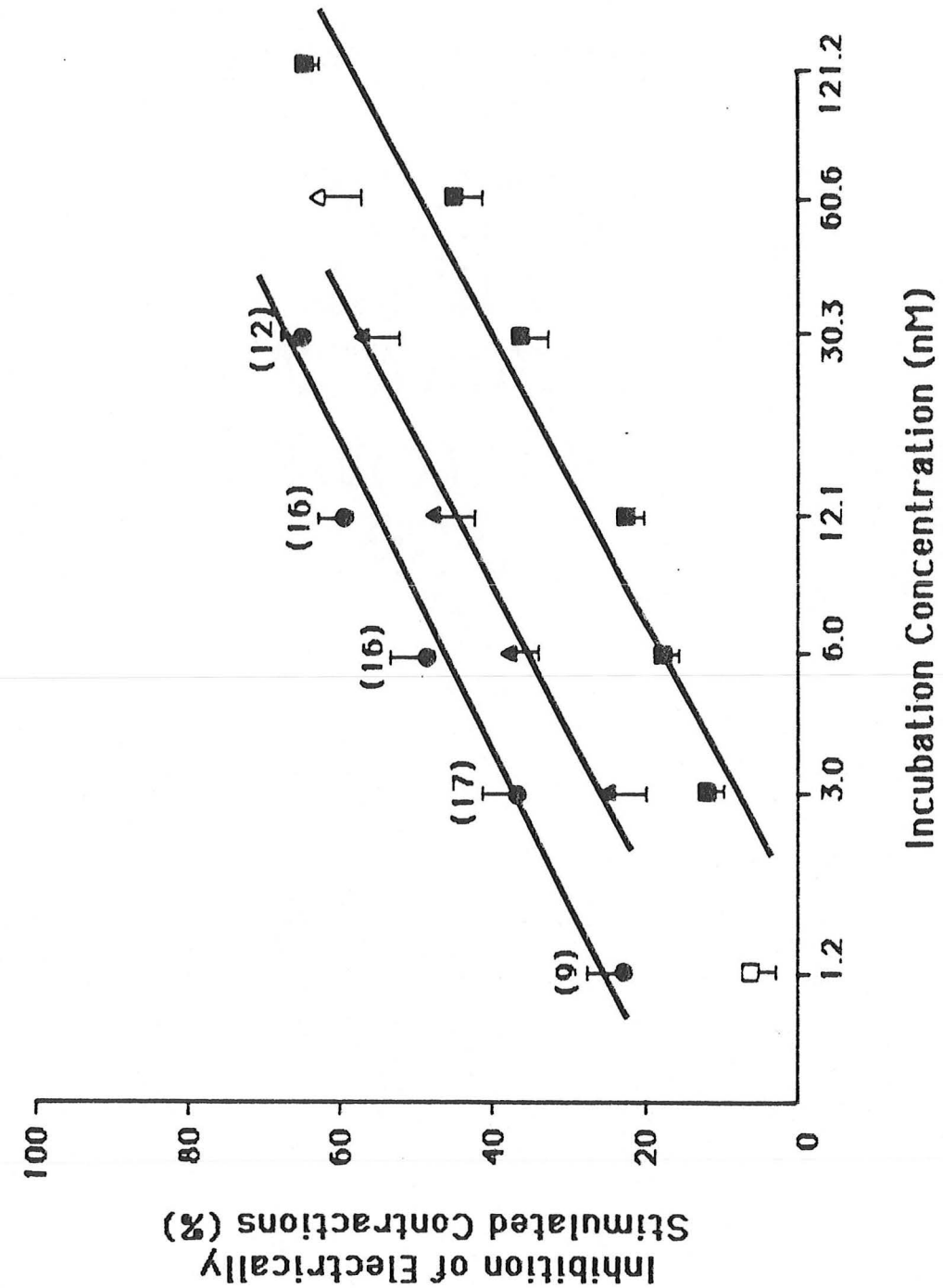

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
1.2
3.0
6.0
12.1
30.3
60.6
121.2
Incubation Concentration (nM)
(9)
(17)
(16)
(16)
(12)

Figure 24. Agonist Capacity of Leu-ENK and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

Leu-ENK (●) 408.0 nM, 43.6 (r = 0.86, N = 84)
Leu-ENK in presence of MRSAL, 4.2 μM (■) 1,449.6 nM, 49.8 (r = 0.85, N = 21)
Leu-ENK in presence of MRSAL, 21.2 μM (▲) 10,937.0 nM, 27.2 (r = 0.92, N = 70)
Leu-ENK in presence of MRSAL, 170.0 μM (▼) 158,489.3 nM, 17.5 (r = 0.89, N = 49)

Clear points (o, △, ▽) were not used in linear progression.

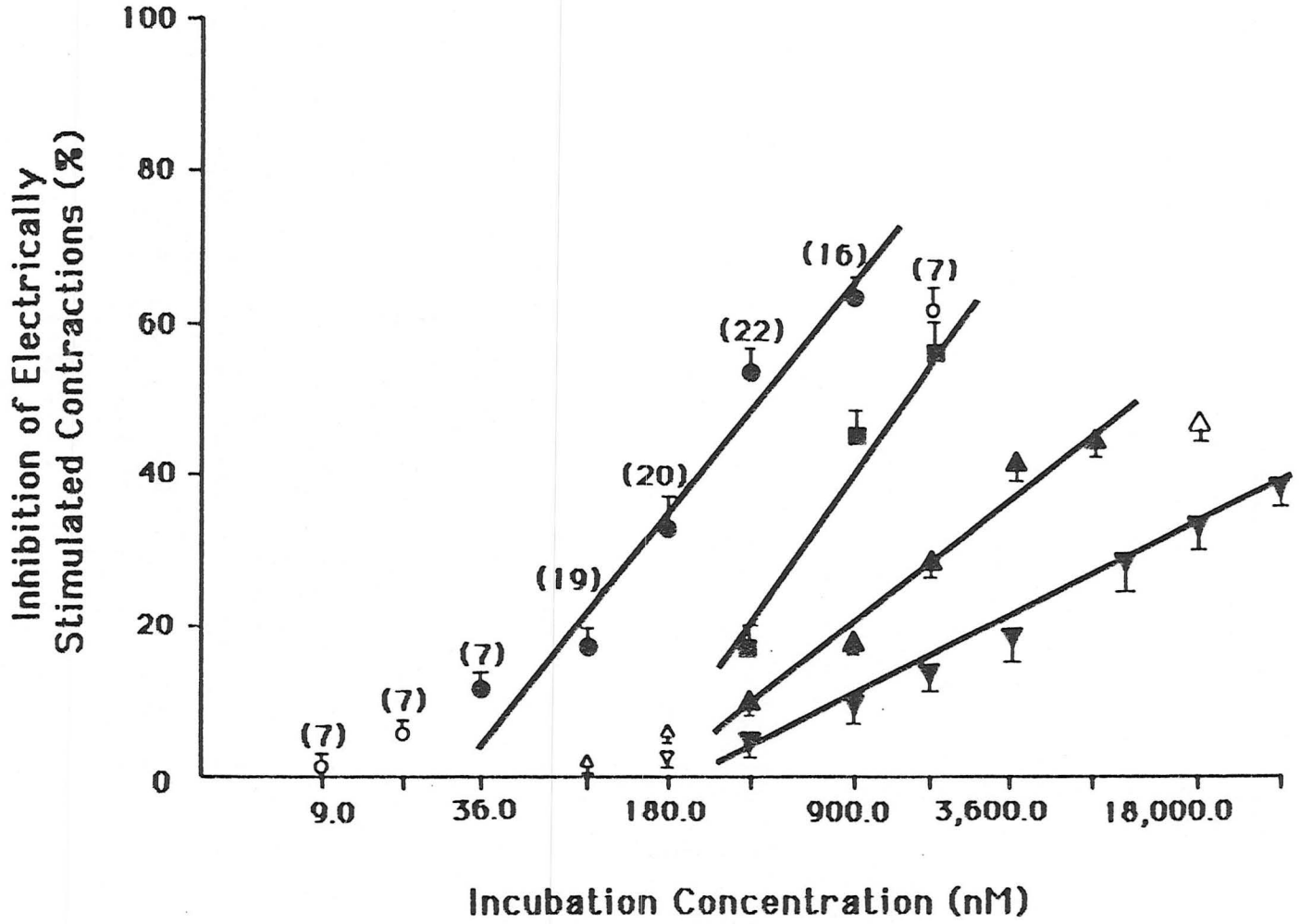

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(7)
(7)
(7)
(19)
(20)
(22)
(16)
(7)
9.0
36.0
180.0
900.0
3,600.0
18,000.0
Incubation Concentration (nM)

Figure 25. Agonist Capacity of Leu-ENK and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

Leu-ENK (●) 19.6 nM, 42.9 (r = 0.91, N = 63)
Leu-ENK in presence of MRSAL, 42.5 μM (▲) 23.6 nM, 37.9 (r = 0.87, N = 24)
Leu-ENK in presence of MRSAL, 170.0 μM (■) 50.1 nM, 39.2 (r = 0.96, N = 36)

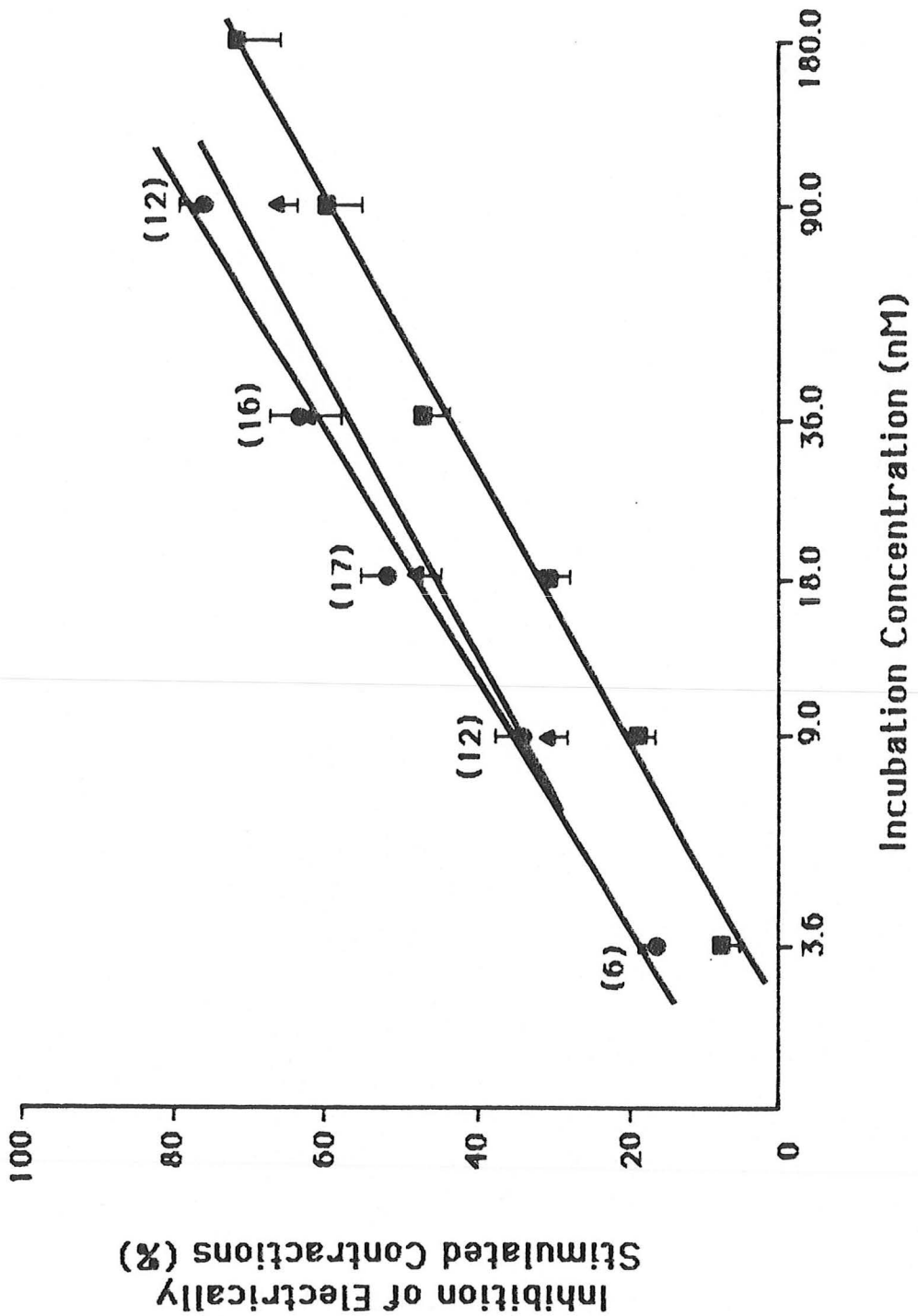

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
3.6
9.0
18.0
36.0
90.0
180.0
Incubation Concentration (nM)
(6)
(12)
(17)
(16)
(12)

Figure 26. Agonist Capacity of DADLE and Its Antagonism by MRSAL in the GPI.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DADLE (●) 19.9 nM, 35.7 (r = 0.93, N = 80)
DADLE in presence of MRSAL, 21, 2 μM (▲) 101.7 nM, 36.4 (r = 0.94, N = 35)
DADLE in presence of MRSAL, 85.0 μM (▼) 210.2 nM, 40.9 (r = 0.96, N = 56)

Clear points (o, △, ▽) were not used in linear regression.

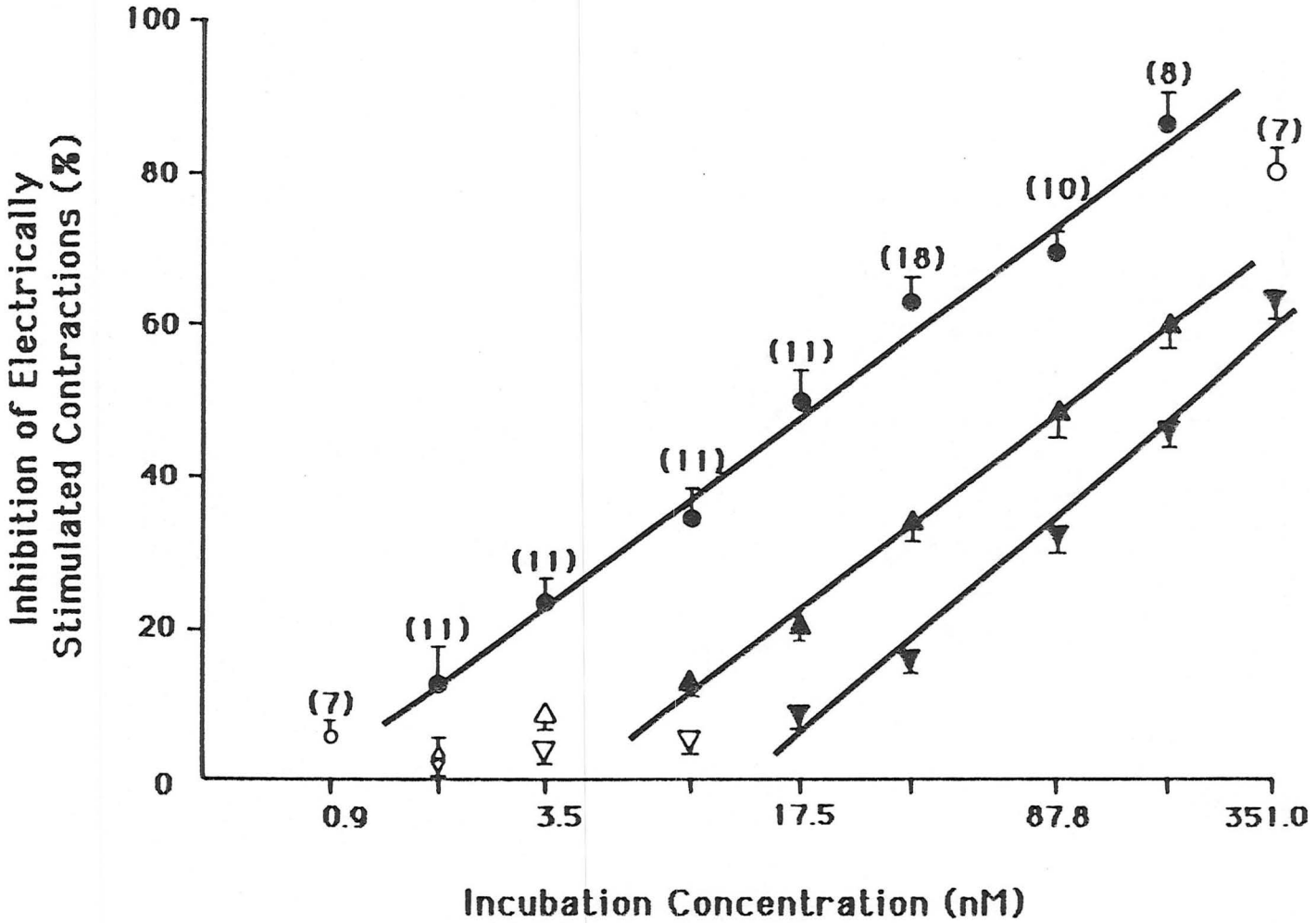
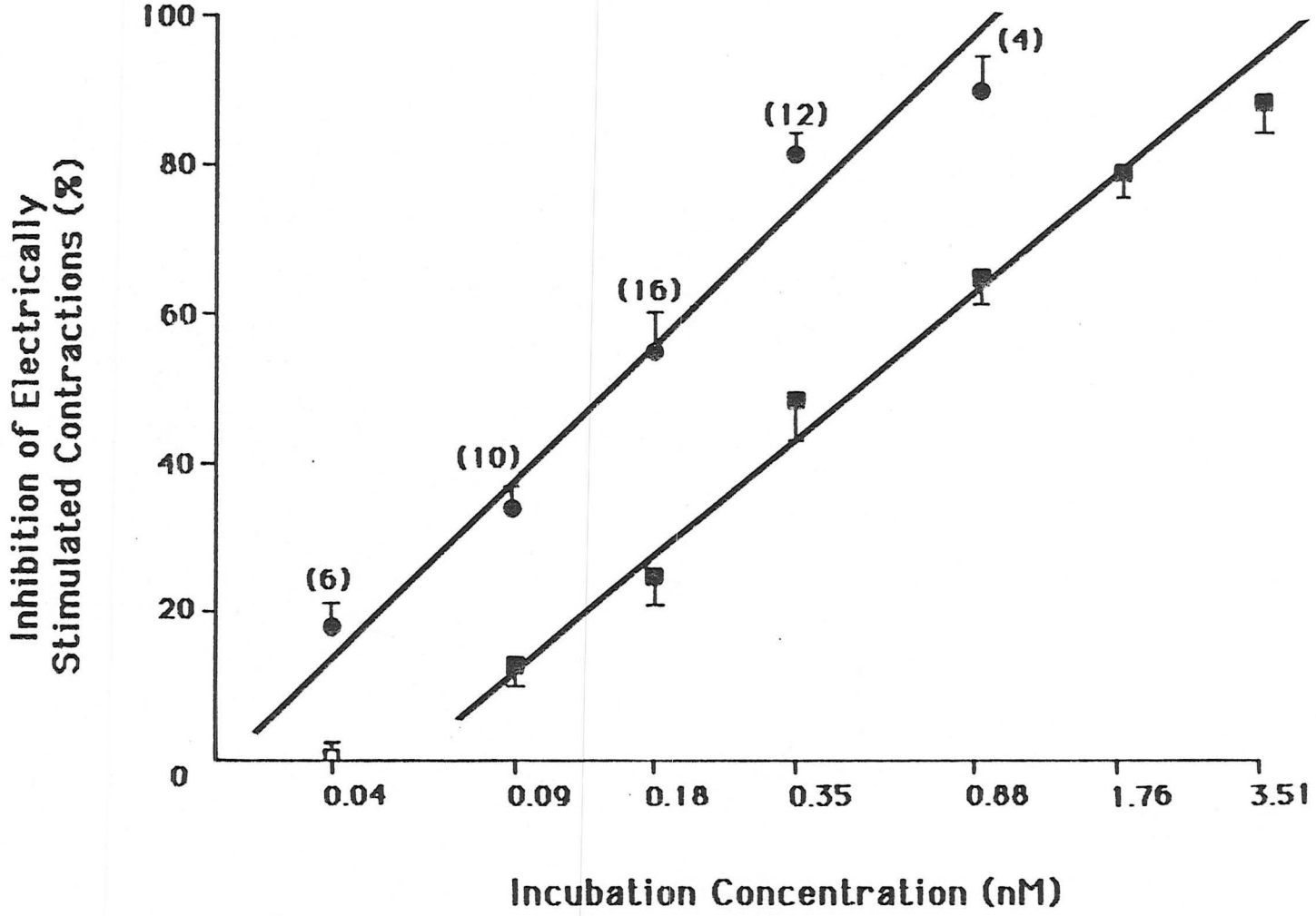

Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(7)
(11)
(11)
(11)
(11)
(18)
(10)
(8)
(7)
0.9
3.5
17.5
87.8
351.0
Incubation Concentration (nM)

Figure 27. Agonist Capacity of DADLE and Its Antagonism by MRSAL in the MVD.

When x = log concentration and y = response, the calculated IC_{50} and slope (b) values are:

DADLE (●) 0.1 nM, 60.5 (r = 0.92, N = 48)
DADLE in presence of MRSAL, 170.0 μM (■) 0.5 nM, 51.5 (r = 0.94, N = 57)

Clear point (□) was not used in linear regression.


Inhibition of Electrically Stimulated Contractions (%)
100
80
60
40
20
0
(6)
(10)
(16)
(12)
(4)
0.04
0.09
0.18
0.35
0.88
1.76
3.51
Incubation Concentration (nM)

against Leu-ENK and B_H-END (Figures 20 and 24). The IC_{50} value for Leu-ENK was increased from 408.0 to 1,449.6 nM in the presence of MRSAL (4.2 μM). This concentration of MRSAL (4.2 μM) was the lowest concentration used for an effective antagonism. The IC_{50} value for B_H-END increased from 37.0 to 228.3 nM in the presence of MRSAL (21.2 μM). In addition, in both cases there was a significant loss of parallelism with higher concentrations of MRSAL. Slope (b) values decreased from 43.6 to 27.2 for Leu-ENK in the presence of MRSAL at concentrations of 21.2 μM (Figure 24), and from 48.5 to 34.5 for B_H-END under the same conditions (Figure 20).

In the MVD, the most effective antagonism with MRSAL was against DHM and NM (Figures 13 and 15). In contrast, the effects of Leu-ENK and B_H-END were resistant to MRSAL antagonism (Figures 21 and 25). Therefore, it was necessary to use MRSAL in concentrations as high as 170 μM in order to obtain some displacement of the concentration-response curve of Leu-ENK to the right. The IC_{50} values of Leu-ENK increased slightly from 19.6 to 39.2 nM (Figure 25). In addition, as shown in Figure 21, the IC_{50} value for B_H-END only increased from 48.4 to 77.3 nM in the presence of MRSAL (85 μM).

MRSAL was also effective against compound J in both preparations. In this case, the antagonism was also reversible and concentration dependent (Figures 9 and 10).

Figures 28 to 43 illustrate the antagonism of MRSAL when this compound was added after the agonists. MRSAL was able to antagonize the decrease in muscular contractions induced by all the opioid agonists tested. The recovery of muscular contractions was concentration-dependent and very fast (30 sec) after MRSAL was added to the bath.

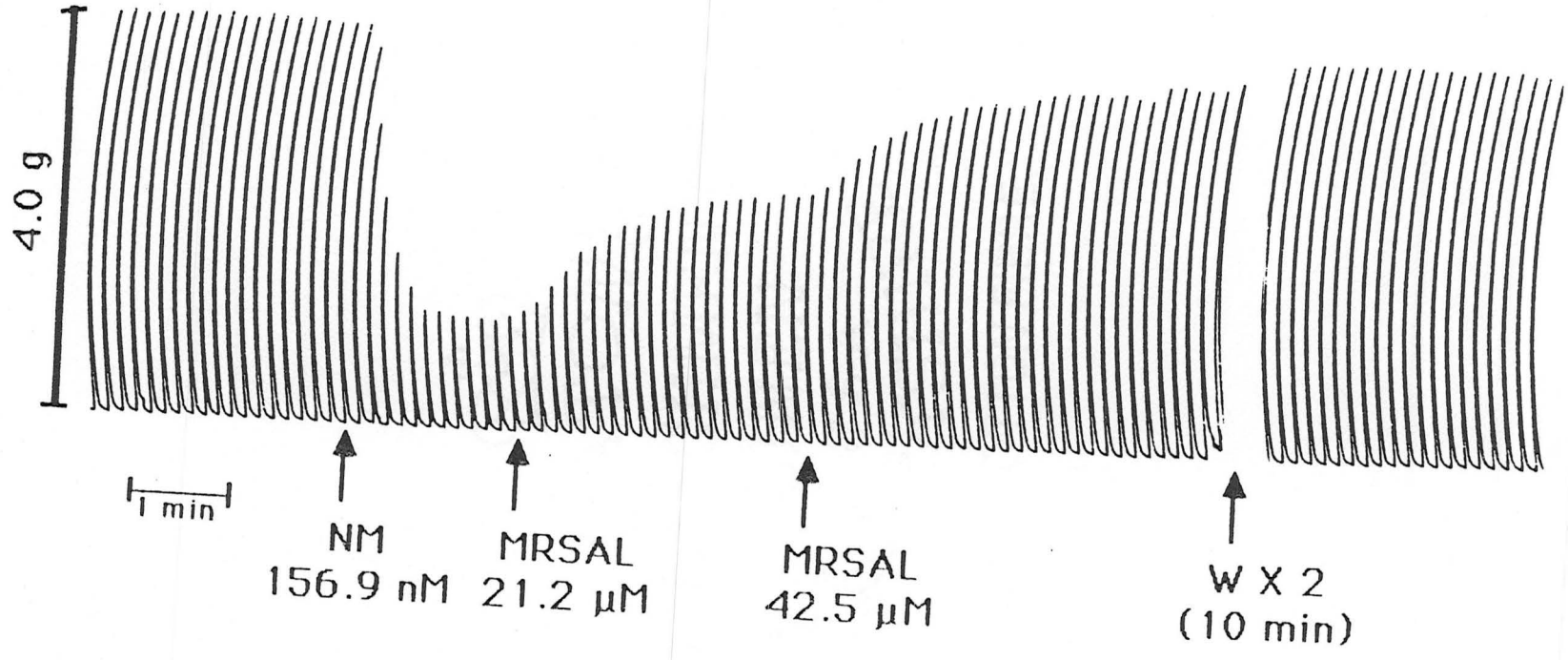


Figure 28. Agonist Activity of NM and Its Antagonism by MRSAL in the GPI.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

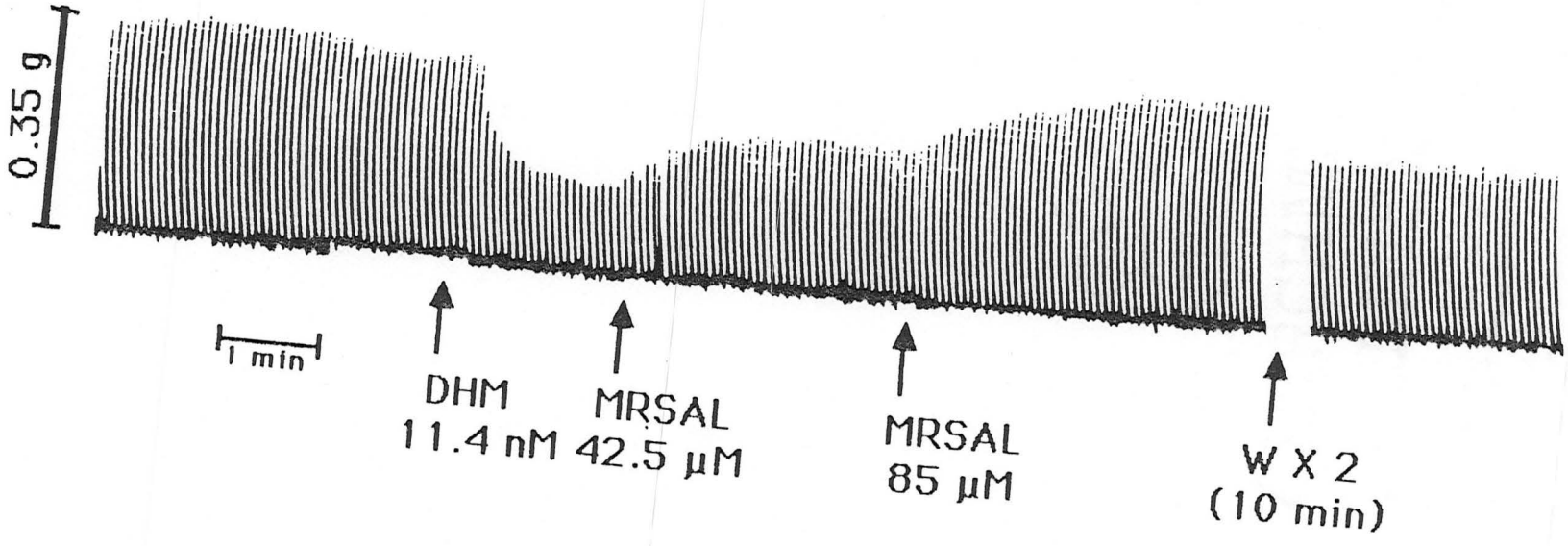


Figure 29. Agonist Activity of DHM and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

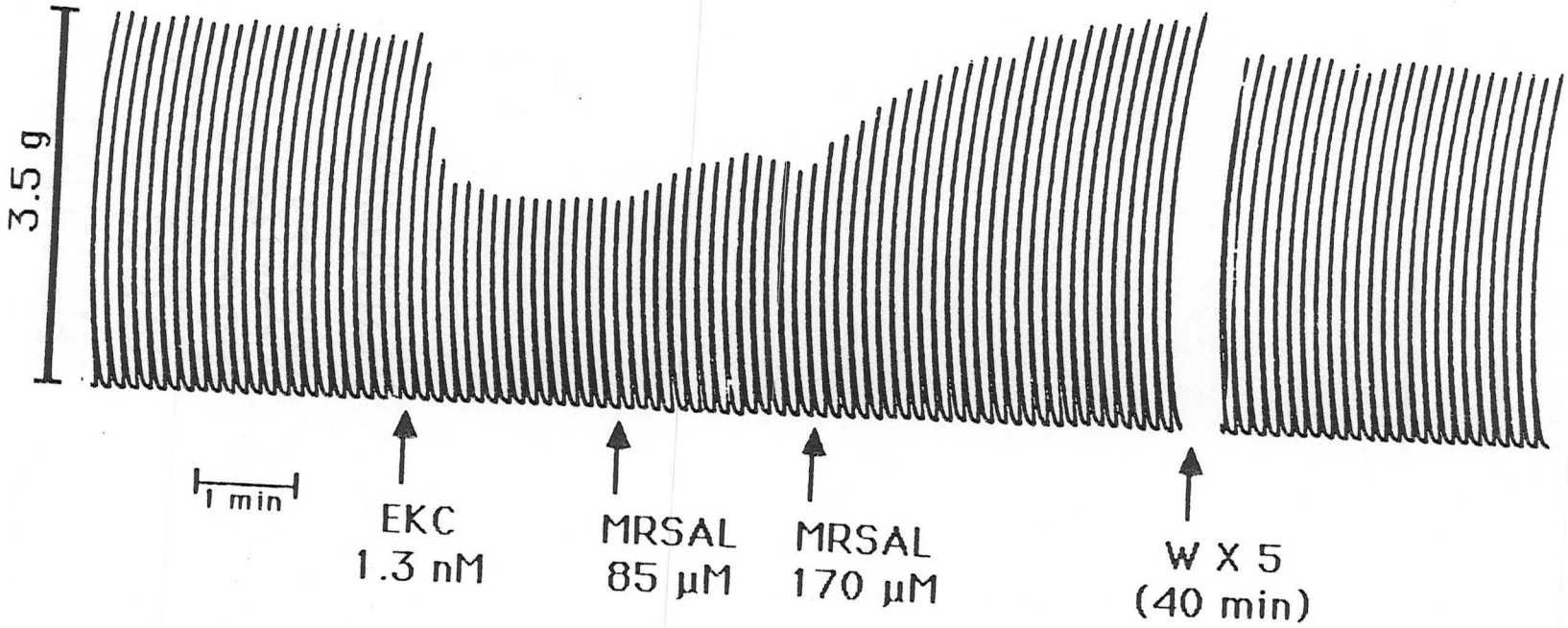


Figure 30. Agonist Activity of EKC and Its Antagonism by MRSAL in the GPI.

W x 5: The preparation was washed 5 times. Muscular contractions were recorded 40 min after wash.

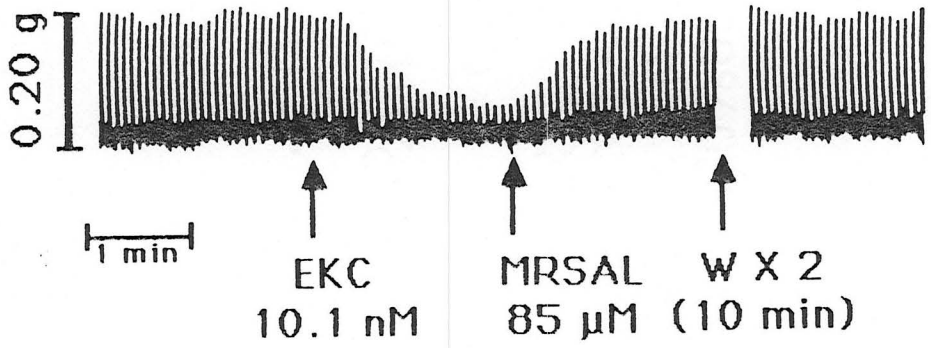


Figure 31. Agonist Activity of EKC and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

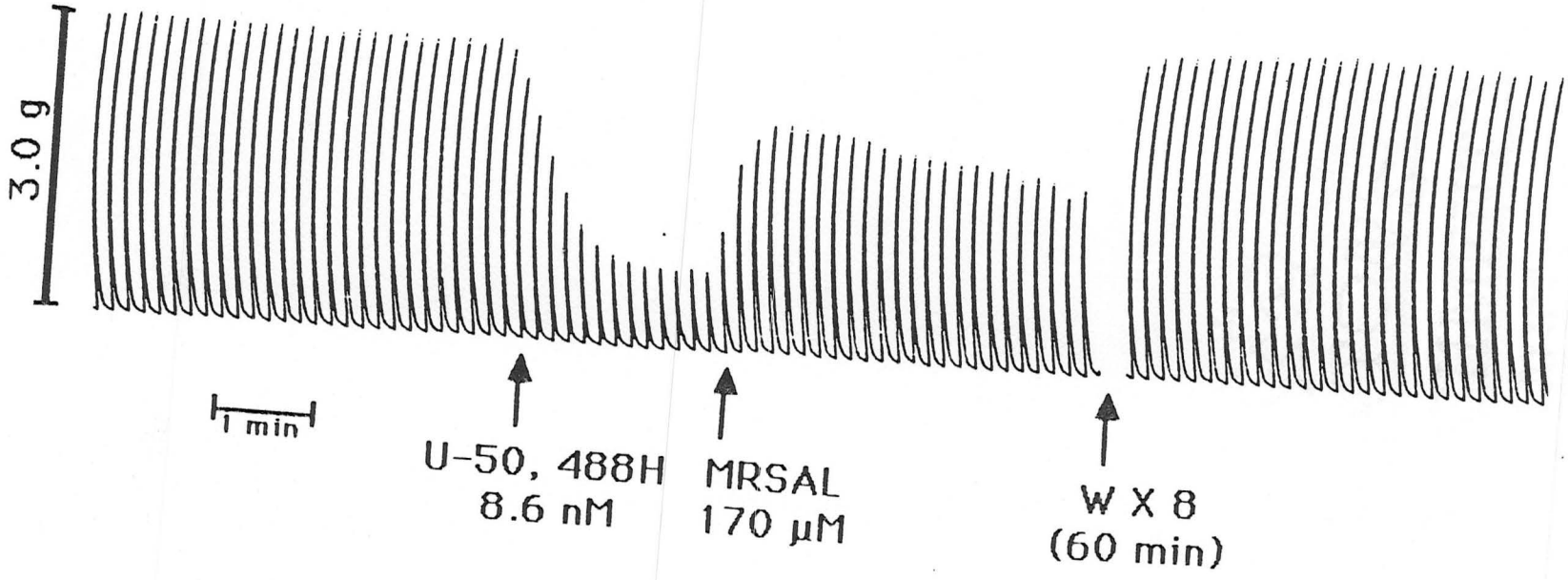


Figure 32. Agonist Activity of U-50,488H and Its Antagonism by MRSAL in the GPI.

W x 8: The preparation was washed 8 times. Muscular contractions were recorded 60 min after wash.

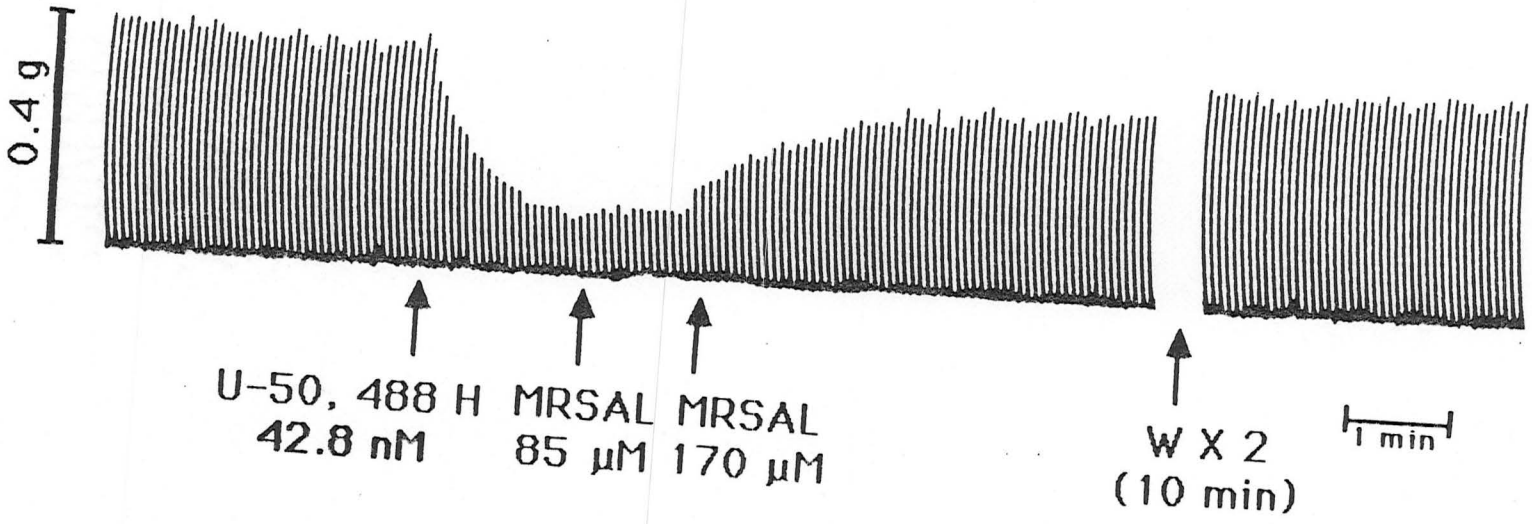


Figure 33. Agonist Activity of U-50,488H and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

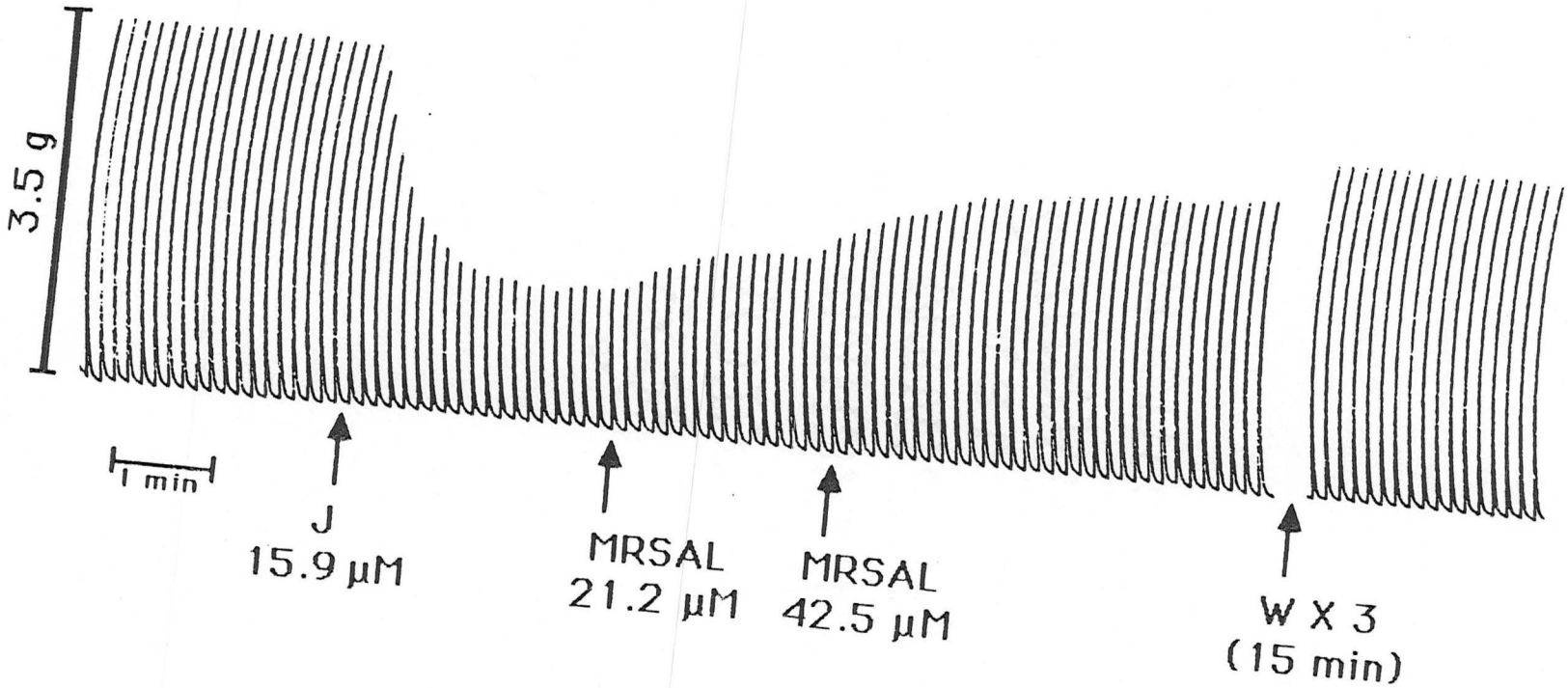


Figure 34. Agonist Activity of J and Its Antagonism by MRSAL in the GPI.

W x 3: The preparation was washed 3 times. Muscular contractions were recorded 15 min after wash.

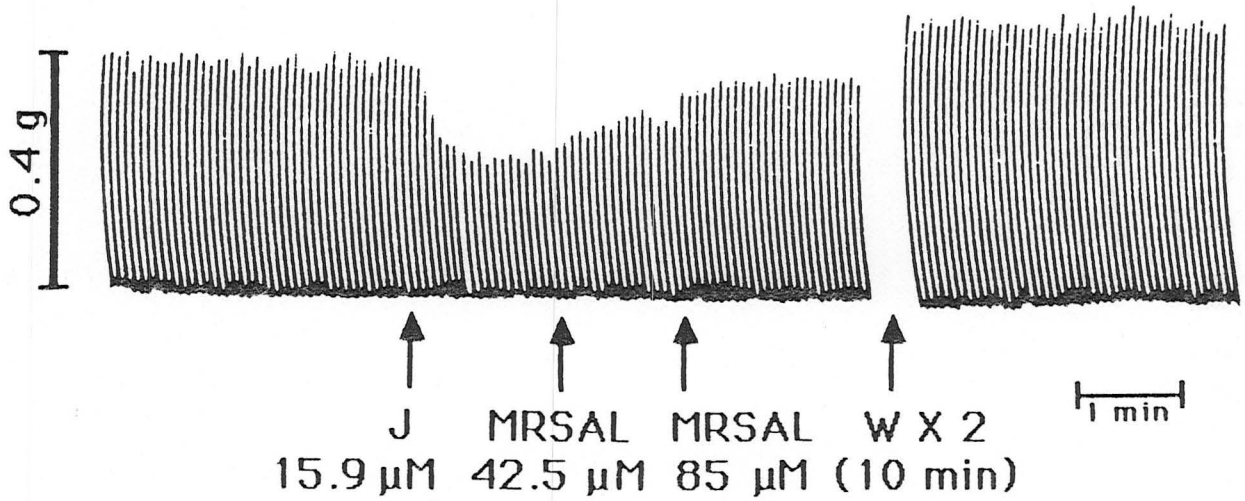


Figure 35. Agonist Activity of J and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

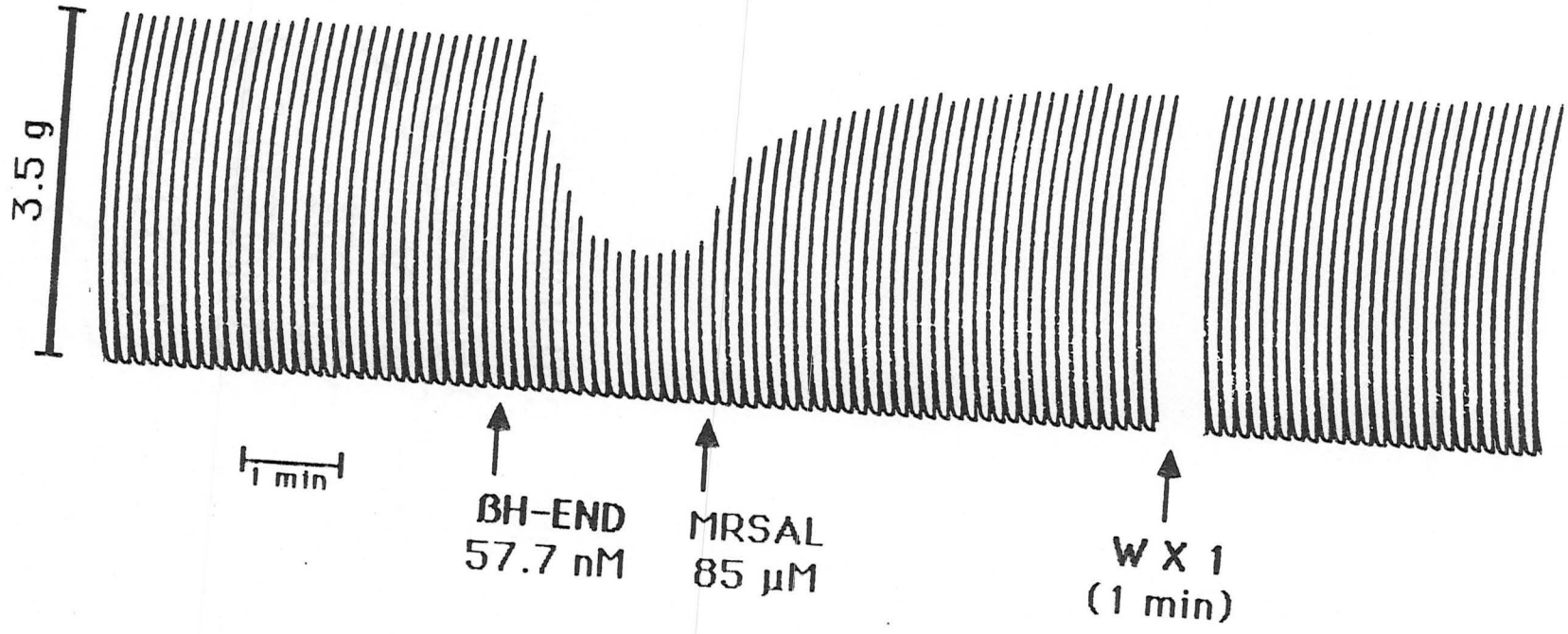


Figure 36. Agonist Activity of β_H-END and Its Antagonism by MRSAL in the GPI.

W x 1: The preparation was washed 1 time. Muscular contractions were recorded within 1 min after wash.

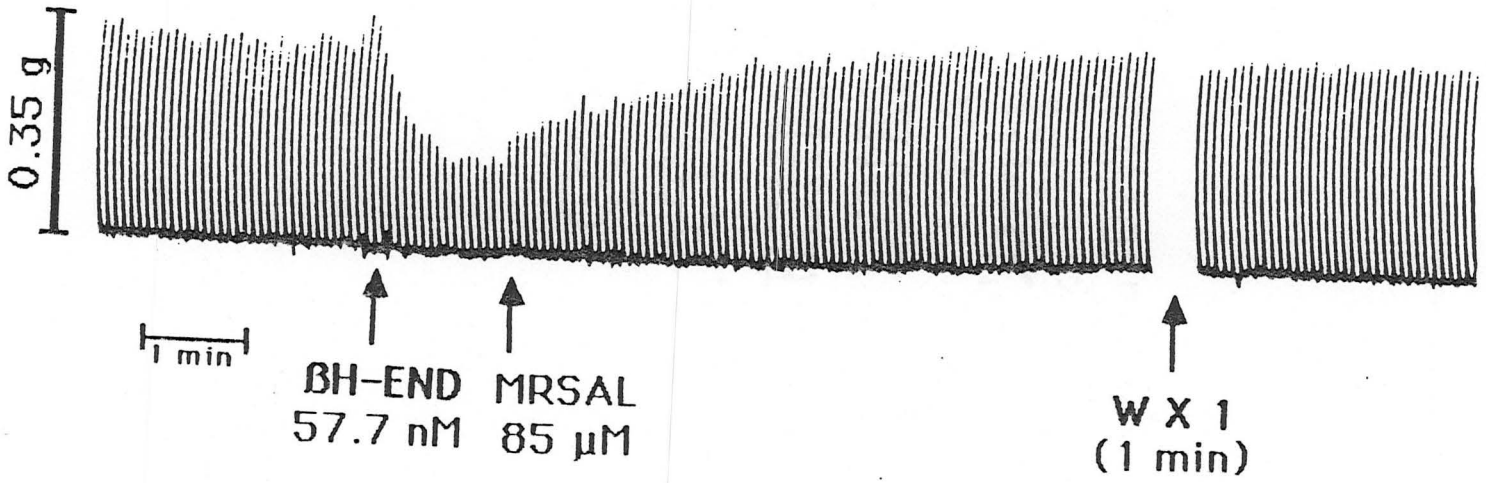


Figure 37. Agonist Activity of B_H-END and Its Antagonism by MRSAL in the MVD.

W x 1: The preparation was washed 1 time. Muscular contractions were recorded within 1 min after wash.

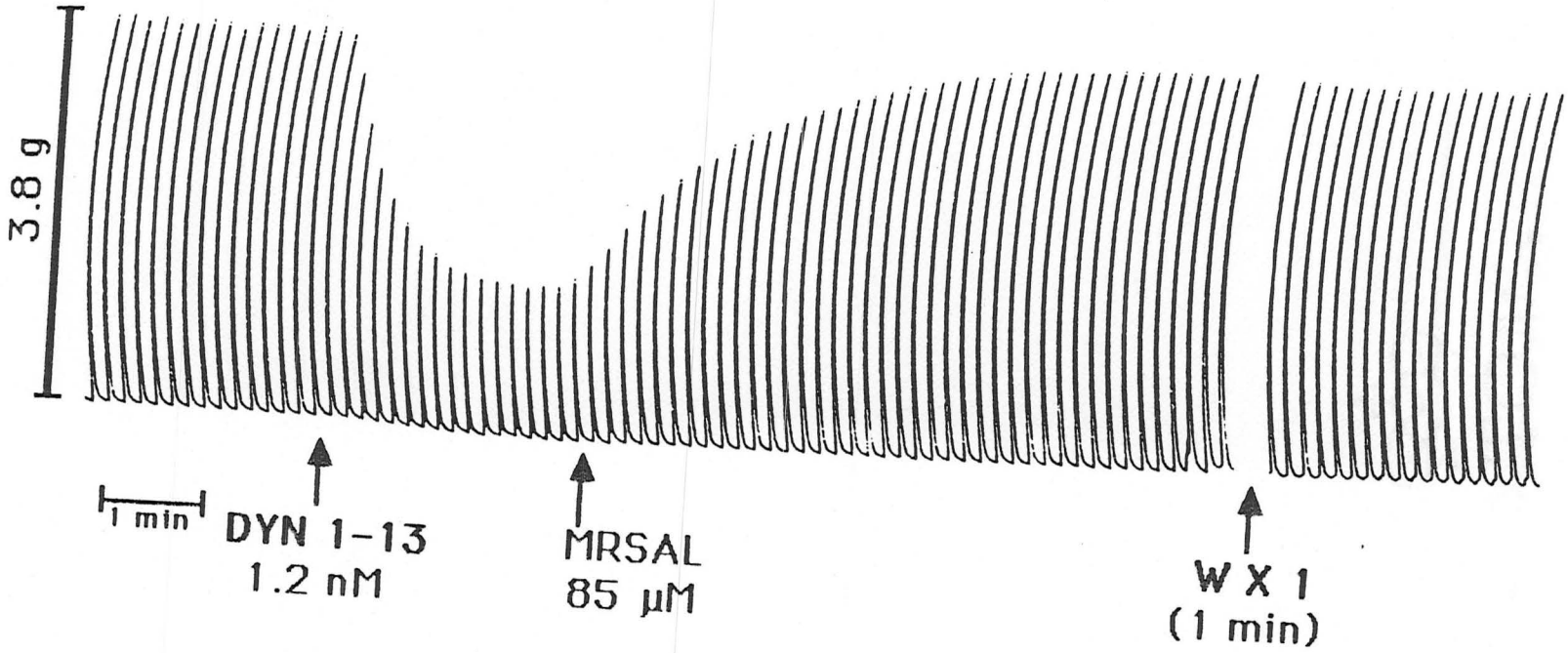


Figure 38. Agonist Activity of DYN 1-13 and Its Antagonism by MRSAL in the GPI.

W x 1: The preparation was washed 1 time. Muscular contractions were recorded within 1 min after wash.

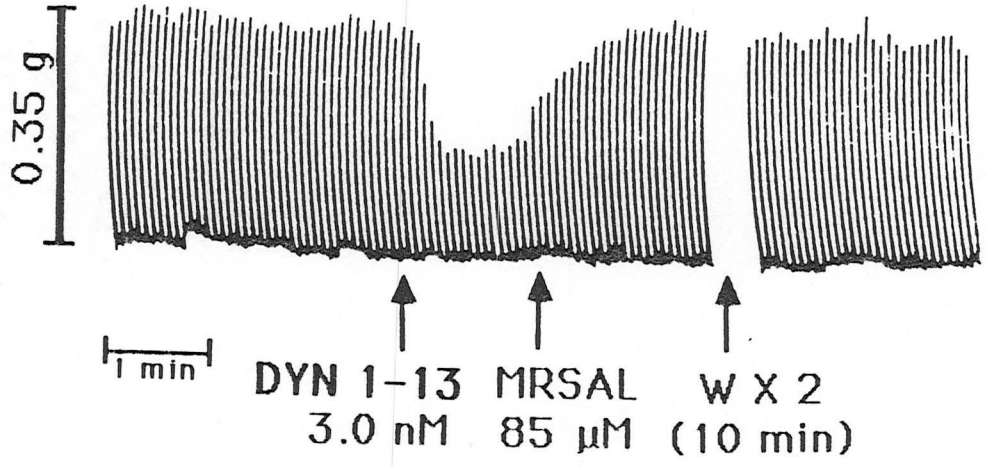


Figure 39. Agonist Activity of DYN 1-13 and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

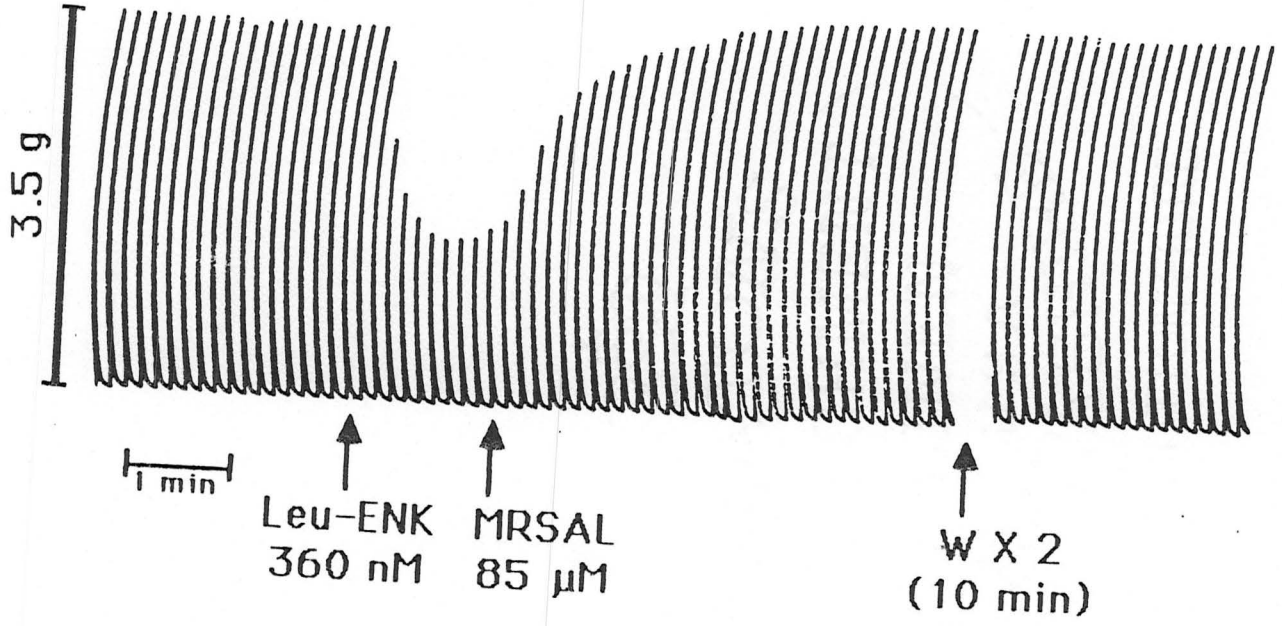


Figure 40. Agonist Activity of Leu-ENK and Its Antagonism by MRSAL in the GPI.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

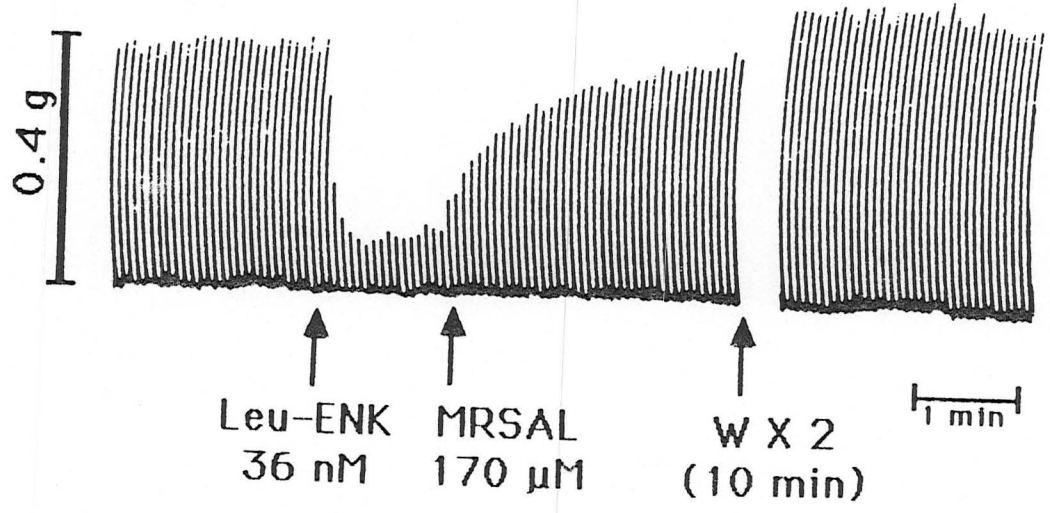


Figure 41. Agonist Activity of Leu-ENK and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

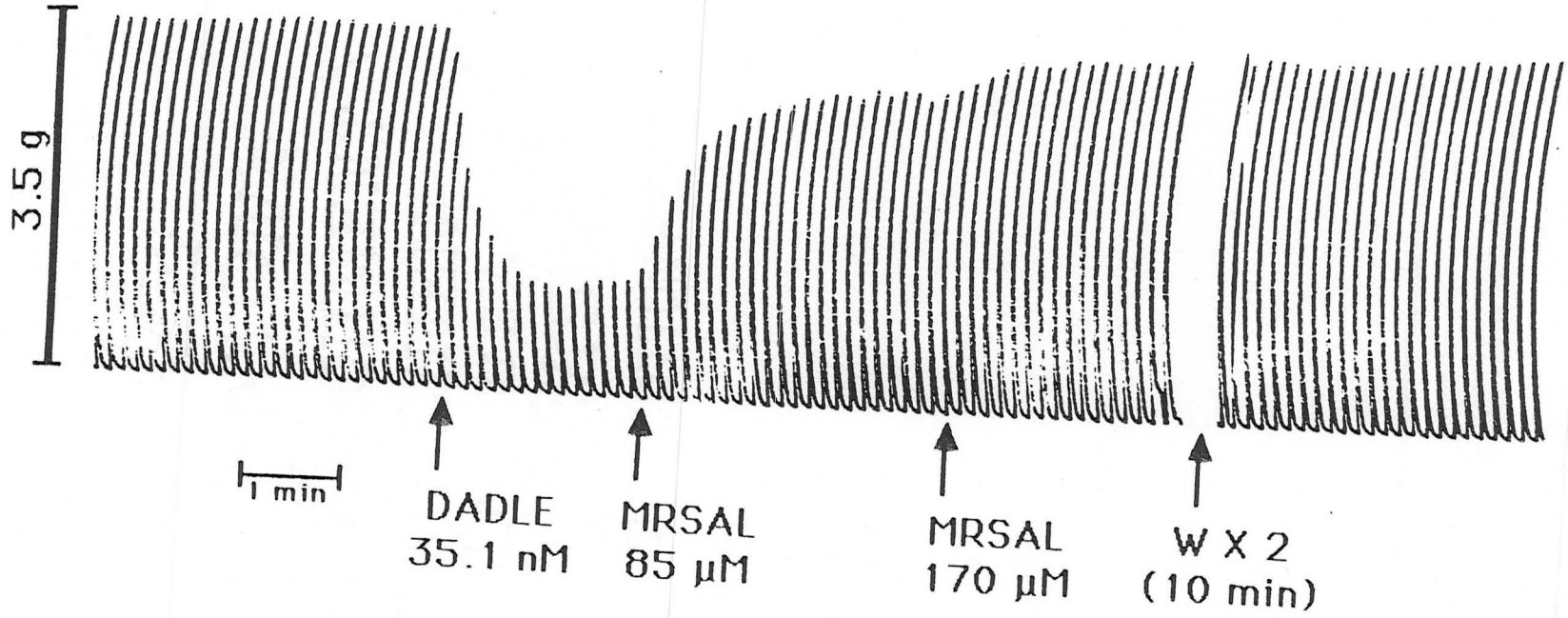


Figure 42. Agonist Activity of DADLE and Its Antagonism by MRSAL in the GPI.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

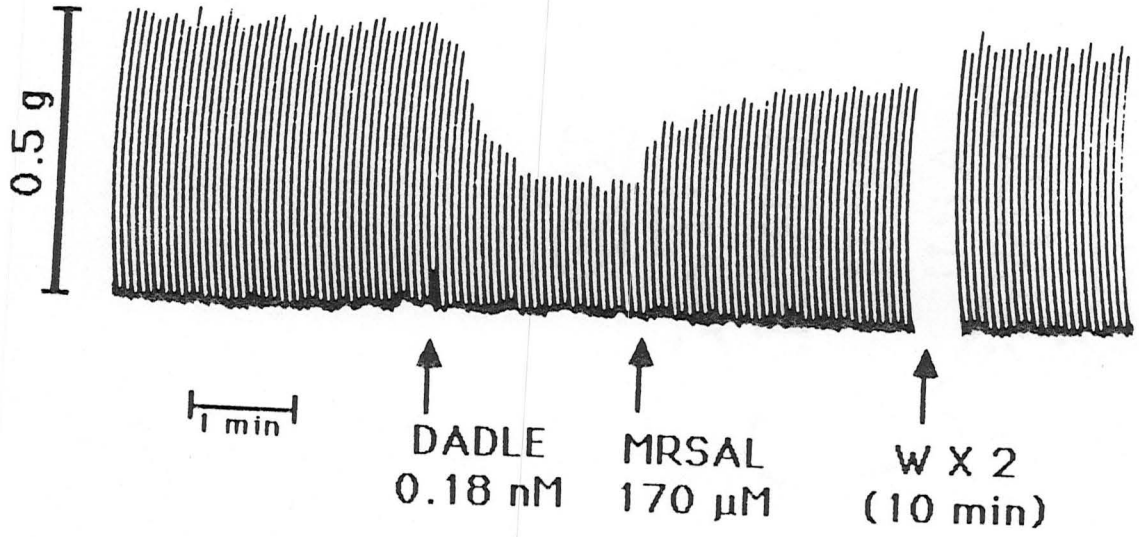


Figure 43. Agonist Activity of DADLE and Its Antagonism by MRSAL in the MVD.

W x 2: The preparation was washed 2 times. Muscular contractions were recorded 10 min after wash.

Qualitatively, the ability of MRSAL to antagonize the effects of the opioids varied among some agonists. NM (mu prototype) was better antagonized in the GPI (Figure 28) than EKC and U-50,488H (Figures 30 and 32). DHM had similar characteristics as NM (not shown). In addition, EKC (kappa prototype) was better antagonized by MRSAL (Figure 30) than U-50,488H, another kappa agonist (Figure 32). The quality of antagonism also differs from GPI to MVD. In general, higher concentrations of MRSAL (42.5-170.0 μM) were used in the MVD for most agonists. DHM (Figure 29) and NM (not shown) had similar behaviors while EKC (Figure 31) was again better antagonized by MRSAL (85 μM) than U-50,488H (Figure 33).

Compound J (Figures 34 and 35) demonstrated more similarities of action to mu prototypes (NM, DHM) than to kappa agonists (EKC and U-50,488H) in both preparations.

The recovery of muscle contraction inhibition induced by the peptide agonists (B_H-END, DYN 1-13, Leu-ENK and DADLE) in the presence of MRSAL in both preparations is shown in Figures 36 to 43. In the GPI, B_H-END (Figure 36) was better antagonized by MRSAL than in the MVD (Figure 37) while DYN 1-13 was equally sensitive to MRSAL antagonism (Figures 38 and 39). In contrast, Leu-ENK and DADLE were far better antagonized in the GPI (Figures 40 and 42) than in the MVD (Figures 41 and 43). In the latter case, the recovery rate was slower and required higher concentrations of MRSAL (170 μM) for antagonism. This subject will be better discussed in a subsequent section titled "Recovery Rate for the Peptides in Guinea Pig Ileum Longitudinal Muscle."

Antagonist-Receptor Affinity of NX, pA_2 Values

The opioid receptor affinity of the prototype antagonist, NX, was established by calculating its pA_2 value against several opioid agonist prototypes and compound J. The calculated pA_2 values for NX antagonism of opioid agonists in the GPI and MVD are shown in Table XII. In both preparations, NX demonstrated its highest affinity for mu receptors (NM and DHM) while its lowest affinity was observed for kappa (EKC, U-50,488H, DYN 1-13) and delta (Leu-ENK and DADLE) receptors. The receptor preference for B_H-END will be fully discussed later.

Compound J was antagonized by NX in the same manner as NM, DHM and Leu-ENK in the GPI. There was no significant difference between the pA_2 values of J, DHM, B_H-END and Leu-ENK. However, there was a small but significant difference ($P < 0.05$) between the pA_2 values for NX antagonism of J, NM and DADLE. In the same preparation, the pA_2 value for NX antagonism of J was very highly significantly different ($P < 0.001$) from that of kappa receptors prototypes like EKC, U-50,488H and DYN 1-13 (Table XII).

In the MVD, the pA_2 value for NX antagonism of J was slightly lower (8.00) than in the GPI (8.17). In this preparation, there was a very highly significant difference ($P < 0.001$) between the pA_2 values of J and DHM, EKC, DYN 1-13, B_H-END, Leu-ENK and DADLE, but not NM (Table XII).

The apparent equilibrium dissociation constant (Ke) of NX for several opioid agonists in the GPI and MVD as reported by other investigators is presented in Tables XIII, XIV and XV. This constant (Ke) measures the effectiveness of an antagonist against a given agonist and represents the concentration of the antagonist which is necessary to

Table XII. Calculated pA2 Values for NX Antagonism of Several Opioid Agonists.

Opioid Agonist	GPI		MVD	
	N	pA_2 ± S.E.	N	pA_2 ± S.E.
NM	15	8.40 ± 0.06*	5	8.12 ± 0.05
DHM	8	8.27 ± 0.02	11	8.48 ± 0.08***
EKC	14	7.62 ± 0.08***	9	7.15 ± 0.07***
U-50,488H	13	7.53 ± 0.09***	10	7.72 ± 0.07*
B_H-END	7	7.87 ± 0.13	13	7.52 ± 0.07***
DYN 1-13	10	7.44 ± 0.05***	13	7.48 ± 0.09***
Leu-ENK	7	8.32 ± 0.06	21	7.41 ± 0.06***
DADLE	7	7.80 ± 0.06*	7	7.03 ± 0.09***
J	30	8.17 ± 0.07	20	8.00 ± 0.08

Level of significance relative to J: * $\underline{P} < 0.05$, *** $\underline{P} < 0.001$.

Table XIII. Apparent Equilibrium Dissociation Constant (Ke) of NX in the GPI as Reported by Other Investigators.

Opioid Agonist	Ke (nM)[1]	pA_2[2] value	Reference
NM	2.64 ± 0.04	8.58	Waterfield et al., 1977
	2.64 ± 0.04	8.58	Lord et al., 1977
	3.5 (2.5-4.7)	8.46	Huidobro-Toro et al., 1982
	3.5 ± 0.5	8.46	Chavkin et al., 1982
	-	8.46 ± 0.22[3]	Huidobro-Toro et al., 1981b
	3.5 (2.5-4.7)	8.46	Yoshimura et al., 1982c
	1.9 ± 0.2	8.72	Hutchinson et al., 1975
	1.9	8.72	Paterson et al., 1984
DHM	2.8 ± 0.2	8.55	Leslie et al., 1980
EKC	14.9 ± 0.9	7.83	Lord et al., 1977
	21.9 (15.6-30.6)	7.66	Huidobro-Toro et al., 1982
	13.0 ± 1.53	7.89	Oka et al., 1982
	23.0 ± 3.7	7.64	Chavkin et al., 1982
	23.8 (17.1-33.1)	7.62	Yoshimura et al., 1982c
	-	7.88 ± 0.10[3]	Takemori and Portoghese, 1984
	21.9 (15.63-30.62)	7.66	Yoshimura et al., 1982a
	-	7.88 ± 0.09[3]	Takemori et al., 1981
	14.9 ± 0.9	7.83	Hutchinson et al., 1975
	15.0	7.82	Paterson et al., 1984
U-50,488H	-[4]		

[1]Ke value reported either as Mean ± S.E. or with its 95% confidence limits.
[2]pA_2 value was calculated from the expression $pA_2 = -\log Ke$ (in molar units).
[3]pA_2 value reported by the investigators as Mean ± S.E.
[4]Not reported in the scientific literature.

Table XIV. Apparent Equilibrium Dissociation Constant (Ke) of NX in the GPI as Reported by Other Investigators.

Opioid Agonist	Ke (nM)[1]	pA_2[2] value	Reference
B-END	2.86 ± 0.42	8.54	Lord et al., 1977
DYN 1-13	27.0 (19.5-37.4)	7.57	Huidobro-Toro et al., 1982
	18.8 ± 2.49	7.73	Oka et al., 1982
	21.0 ± 1.70	7.68	Chavkin et al., 1982
	22.4 (16.98-29.51)	7.65	Yoshimura et al., 1982a
	27.0 (19.5-37.4)	7.57	Yoshimura et al., 1982c
	25.0	7.60	Paterson et al., 1984
Leu-ENK	2.43 ± 0.67	8.61	Waterfield et al., 1977
	2.43 ± 0.67	8.61	Lord et al., 1977
	4.8 (3.3-6.9)	8.32	Huidobro-Toro et al., 1982
	4.4 ± 0.8	8.36	Chavkin et al., 1982
	4.8 (3.33-6.89)	8.32	Yoshimura et al., 1982a
	5.0 (3.7-6.9)	8.30	Yoshimura et al., 1982c
	2.4	8.62	Paterson et al., 1984
DADLE	-	8.51 ± 0.12[3]	Takemori and Portoghese, 1985b
	2.6 ± 0.1	8.59	Leslie et al., 1980

[1]Ke value reported either as Mean ± S.E. or with its 95% confidence limits.
[2]pA_2 value was calculated from the expression pA_2 = -log Ke (in molar units).
[3]pA_2 value reported by the investigators as Mean ± S.E.

Table XV. Apparent Equilibrium Dissociation Constant (Ke) of NX in the MVD as Reported by Other Investigators.

Opioid Agonist	Ke (nM)[1]	pA_2[2] value	Reference
NM	1.84 ± 0.20	8.74	Waterfield et al., 1977
	1.84 ± 0.20	8.74	Lord et al., 1977
	1.20	8.92	Schulz et al., 1984
	3.1 ± 0.3	8.51	Hutchinson et al., 1975
	3.1	8.51	Paterson et al., 1984
DHM	2.6 ± 0.2	8.59	Leslie et al., 1980
EKC	11.0 ± 0.6	7.96	Lord et al., 1977
	15.2 ± 1.7	7.82	Oka et al., 1982
	11.0 ± 0.6	7.96	Hutchinson et al., 1975
	15.2 ± 1.7	7.82	Kajiwara et al., 1986
	11.0	7.96	Paterson et al., 1984
U-50,488H	-[4]		
B_H-END	21.7 ± 1.2	7.66	Lord et al., 1977
DYN 1-13	36.5 ± 4.7	7.44	Oka et al., 1982
	30.5	7.52	Schulz et al., 1984
	16.0	7.80	Paterson et al., 1984
Leu-ENK	21.4 ± 3.3	7.67	Waterfield et al., 1977
	21.4 ± 3.3	7.67	Lord et al., 1977
	21.0	7.68	Paterson et al., 1984
DADLE	27.6	7.56	Schulz et al., 1984
	-	7.39 ± 0.21[3]	Takemori and Portoghese, 1984
	25.6 ± 2.4	7.59	Kajiwara et al., 1986
	27.3 ± 4.8	7.56	Leslie et al., 1980

[1]Ke value reported as Mean ± S.E.
[2]pA_2 value was calculated from the expression $pA_2 = -\log Ke$ (in molar units).
[3]pA_2 value reported by the investigators as Mean ± S.E.
[4]Not reported in the scientific literature.

double the concentration of the agonist to give the same pharmacological effect obtained in the absence of the antagonist (Lord, et al., 1977). The effectiveness of an antagonist against an agonist may also be expressed as pA_2 value which is the negative logarithm of Ke. Therefore, the pA_2 value was calculated from the expression pA_2 = log Ke (in molar units) and it is also presented (Tables XIII, XIV and XV) in addition to the Ke values.

In general, the pA_2 values obtained in this study agree with those reported in the literature (Tables XII, XIII, XIV and XV). However, in the GPI, the pA_2 values for NX antagonism of B_H-END and DADLE found in this research were lower (7.87 and 7.80) than those reported (Table XIV). A similar situation was observed in the MVD where pA_2 values for NX antagonism of NM, EKC and DADLE were lower (8.12, 7.15 and 7.03, respectively) than those previously reported by others (Table XV).

Antagonist-Receptor Affinity of MRSAL, pA_2 Values

The opioid receptor affinity for compound MRSAL was assessed by calculating its pA_2 value against several opioid prototypes and compound J (Table XVI). In the GPI, MRSAL demonstrated greater affinity for Leu-ENK and DADLE receptors, the mu receptor (NM, DHM), B_H-END and compound J receptors. Although MRSAL showed less affinity for the kappa sites when assessed using EKC and U-50,488H as prototypes selective for this receptor, its pA_2 value against the DYN-13 opioid receptor was much higher (5.10) than those against EKC (4.50) and U-50,488H (4.77) and comparable to mu prototypes.

In the MVD (Table XVI), the highest affinity was observed toward the mu-receptor (NM, DHM) and compound J. Lower pA_2 values were

Table XVI. Calculated pA2 Values for MRSAL Antagonism of Several Opioid Agonists.

Opioid Agonist	GPI		MVD	
	N	pA2 ± S.E.	N	pA2 ± S.E.
NM	28	5.41 ± 0.02	17	5.10 ± 0.07
DHM	21	5.53 ± 0.05	12	5.22 ± 0.13
EKC	25	4.50 ± 0.07	12	4.35 ± 0.12
U-50,488H	22	4.77 ± 0.07	12	4.90 ± 0.07
B_H-END	17	5.23 ± 0.03	15	4.50 ± 0.06
DYN 1-13	24	5.10 ± 0.05	20	4.98 ± 0.09
Leu-ENK	20	5.71 ± 0.05	12	4.05 ± 0.08
DADLE	20	5.22 ± 0.03	12	4.11 ± 0.11
J	20	5.09 ± 0.04	18	5.12 ± 0.08

obtained for MRSAL antagonism of U-50,488H, B_H-END, EKC and DYN 1-13. A much lower capacity of antagonism was observed against DADLE and Leu-ENK in this preparation, but not in the GPI.

Antagonist Potency, Ke Values for NX and MRSAL in the GPI and MVD

The opioid antagonist potency of NX and MRSAL against several opioid agonists is shown in Table XVII. In the GPI, there was close agreement between the Ke values found in this study and those reported by other investigators (Tables XIII and XIV). One exception was seen with the Ke value for NX against B_H-END. We found a slight decrease in potency for NX antagonism against this peptide (Ke = 17.8 nM) as compared to that of 2.86 nM reported by Lord et al. (1977) (Table XIV). NX was more potent in antagonizing the effects of NM, Leu-ENK, DHM and J than DADLE and B_H-END. A decrease of antagonist potency was further observed when NX was tested against EKC, U-50,488H and DYN 1-13.

In the MVD, highest antagonist potency for NX was seen against DHM, NM and J. NX demonstrated a wide range of antagonist potency when tested against U-50,488H, B_H-END, DYN 1-13, Leu-ENK and EKC (Table XVII). This antagonist was more potent against U-50,488H than against DYN 1-13 or EKC, but demonstrated similar antagonist potency against B_H-END, DYN 1-13 and Leu-ENK. The lowest antagonist potency of NX was seen against DADLE with a Ke value of 103.5 nM (Table XVII). In this study, the Ke values for NX antagonism in the MVD against NM and Leu-ENK were higher than those reported by others. In addition, even higher values were obtained when NX was tested against EKC and DADLE (Tables XV, XVII).

In contrast to NX, MRSAL demonstrated its highest antagonist potency against Leu-ENK, DHM and NM in the GPI and DHM, NM and J in the

Table XVII. Antagonist Potency, Ke Values[1] for NX and MRSAL Against Several Opioid Agonists.

Opioid Agonist	GPI[2]				MVD[2]			
	N	NX (nM)	N	MRSAL (μM)	N	NX (nM)	N	MRSAL (μM)
NM	15	4.5 ± 0.5	28	4.0 ± 0.2	15	8.3 ± 0.9	17	9.6 ± 1.5
DHM	8	5.5 ± 0.3	21	3.5 ± 0.6	11	4.0 ± 0.8	12	9.1 ± 2.5
EKC	14	30.2 ± 6.8	25	42.6 ± 7.1	9	78.5 ± 12.7	12	13.0 ± 1.9
U-50,488H	13	37.0 ± 6.9	22	23.7 ± 4.8	10	21.4 ± 4.4	12	13.0 ± 1.9
B_H-END	7	17.8 ± 5.4	17	6.1 ± 0.4	13	35.1 ± 5.3	15	37.4 ± 5.8
DYN 1-13	10	39.8 ± 7.0	24	8.9 ± 0.9	13	42.4 ± 8.1	20	15.0 ± 2.9
Leu-ENK	7	5.0 ± 0.5	20	2.2 ± 0.2	21	46.2 ± 6.1	12	104.9 ± 15.7
DADLE	7	17.0 ± 2.2	20	6.4 ± 0.5	7	103.5 ± 17.8	12	128.6 ± 57.5
J	30	9.4 ± 1.3	20	9.0 ± 0.1	20	13.8 ± 2.4	18	10.5 ± 3.0

[1]Ke value was calculated from the expression $pA_2 = -\log Ke$ (in molar units).

[2]Values are expressed as Mean ± S.E.

MVD (Table XVII). Leu-ENK was particularly more sensitive to MRSAL antagonism (2.2 μM) in the GPI than any other agonist tested. Intermediate antagonist potency of MRSAL was seen against the agonists B_H-END, DADLE, DYN 1-13 and J in the GPI and EKC, U-50,488H and DYN 1-13 in the MVD. Among these agonists, DYN 1-13 was more sensitive to this antagonist (8.9 μM) than U-50,488H (23.7 μM) and EKC (42.6 μM) in the GPI. In contrast to the GPI, a decrease in antagonist potency was further observed in the MVD when MRSAL was tested against B_H-END, Leu-ENK and DADLE. In the latter preparation, MRSAL displayed very low potency of antagonism against Leu-ENK (104.9 μM) and DADLE (128.6 μM) (Table XVII).

In both preparations, J was more sensitive to NX and MRSAL antagonism than kappa and delta prototypes.

Recovery Rate of Muscular Contraction for the Peptides in the GPI

The recovery rates for muscular contraction for B_H-END, DYN 1-13 and Leu-ENK were calculated in the absence and presence of the antagonist MRSAL in the GPI (Tables XVIII, XIX, XX). The spontaneous recovery of inhibition at 5 min in the absence of MRSAL was slowest for B_H-END (13%, Table XVIII), intermediate for DYN 1-13 (36%, Table XIX) and fastest for Leu-ENK (66%, Table XX).

In general, there was a significant difference ($P < 0.001$) in the recovery rate of these peptides in the presence of MRSAL as compared to control at the same time interval. MRSAL (4.2 - 84.0 μM), significantly increased the recovery rate for B_H-END (Table XVIII), DYN 1-13 (Table XIX) and Leu-ENK (Table XX) at 5, 10 and 15 min. However, there was no significant difference in the recovery rate for DYN-13 in the presence

Table XVIII. Recovery Rate of Muscular Contractions for B_H-END in the Absence and Presence of MRSAL on GPI.

MRSAL (μM)	N	Recovery Rate (%)[1]			
		5 min	10 min	15 min	20 min
0	4	13.2 ± 2.3	49.5 ± 1.9	71.6 ± 2.2	93.4 ± 1.2
4.2	4	89.2 ± 2.6*	98.5 ± 1.5*	-	-
42.5	7	94.3 ± 2.6*	-	-	-

[1]Results are expressed as Mean ± S.E.

*Significantly different from control at the same time interval, $P < 0.001$.

Table XIX. Recovery Rate of Muscular Contractions for DYN 1-13 in the Absence and Presence of MRSAL in the GPI.

MRSAL (μM)	N	Recovery Rate (%)[1]			
		5 min	10 min	15 min	20 min
0	20	36.0 ± 2.5	77.7 ± 3.4	96.2 ± 1.7	98.2 ± 2.8
0.2	7	34.6 ± 4.5	80.0 ± 3.0	97.6 ± 2.4	-
0.8	4	36.0 ± 2.1	72.8 ± 2.0	90.8 ± 2.3	-
8.5	4	45.8 ± 1.6	80.9 ± 2.0	101.2 ± 1.2	-
21.2	8	64.4 ± 3.5*	99.8 ± 2.0*	120.0 ± 5.3*	-
42.5	6	85.2 ± 1.5*	106.8 ± 1.2*	-	-
85.0	10	107.6 ± 4.4*	-	-	-

[1]Results are expressed as Mean ± S.E.

*Significantly different from control at the same time interval, $P < 0.001$.

Table XX. Recovery Rate of Muscular Contractions for Leu-ENK in the Absence and Presence of MRSAL in the GPI

MRSAL (μM)	N	Recovery Rate (%)[1] 5 min
0	11	66.0 ± 1.9
21.2	7	92.6 ± 1.9*
85.0	3	109.2 ± 0.3*

[1]Results are expressed as Mean ± S.E.

*Significantly different from control at the same time interval, $\underline{P} < 0.001$.

of MRSAL in concentrations from 0.2 to 8.5 μM at the same time intervals (Table XIX).

Acute Tolerance Induced by DYN 1-13 in the GPI

DYN 1-13 produced acute tolerance in the GPI when using both the "single-dose" (Fig. 44) and the "cumulative" (Fig. 45) methods of documenting a dose-response curve and also when the same concentration of the peptide was repeated during 130 min (Table XXI). However, this effect seemed more pronounced when DYN 1-13 was tested using the "single-dose" method with a 25 min interval between doses (Fig. 44). There was a higher level of significance ($P < 0.001$) between the first and second concentration-effect curves when using the "single-dose" than when the "cumulative" dose-response method (Fig. 45) was used. When the same concentration of DYN 1-13 was repeated during 130 min at 30-40 min intervals, the effect of acute tolerance induced by this peptide was apparent statistically at 30 min (Table XXI).

B_H-END, Leu-ENK and DADLE did not induce acute tolerance in the GPI. Concentration-response curves for these peptides were reproducible for a period up to 5 h and even after antagonism experiments with MRSAL. This further demonstrates that MRSAL can be "washed out" well from the preparation.

Although it seemed that the spontaneous recovery rate for DYN 1-13 increased with the development of tolerance, there was no significant difference between the recovery rates of muscular contraction at 0 min and 120 min later in the same preparation (not shown).

Figure 44. Acute Tolerance Effect Induced by DYN 1-13 on the GPI When Using "Single-Dose" Method.

Circles (o) correspond to the first DYN 1-13 concentration-effect curve, while squares (□) represent the second DYN 1-13 concentration-effect curve as described in Methods.

Significance level: $P < 0.01$ (**) and $P < 0.001$ (***).

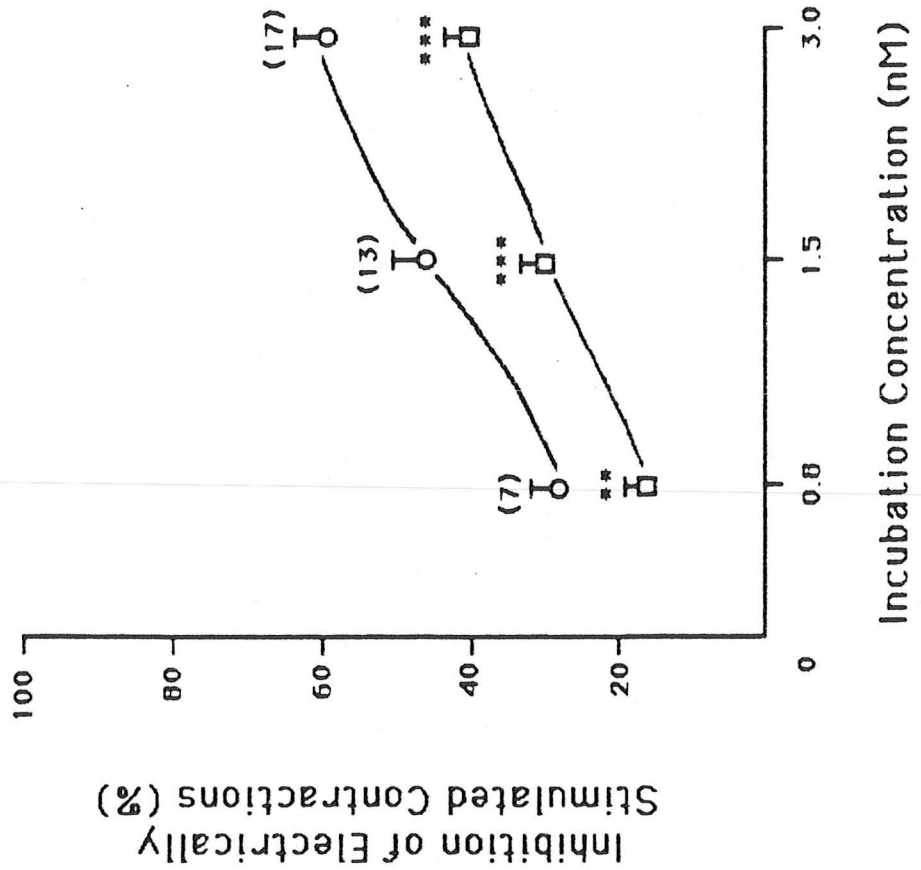

Inhibition of Electrically
Stimulated Contractions (%)
100
80
60
40
20
0
0.8
1.5
3.0
Incubation Concentration (nM)
(7)
(13)
(17)
**
***
***

Figure 45. Acute Tolerance Effect Induced by DYN 1-13 on the GPI When Using Cumulative Method.

Circles (o) represent the first DYN 1-13 concentration-effect curve, while squares (□) represent the second DYN 1-13 concentration-effect curve 60-80 min later (n = 14).

Significance level: $\underline{P} < 0.05$ (*) and $\underline{P} < 0.001$ (***).

NS = no significant difference ($\underline{P} < 0.05$).

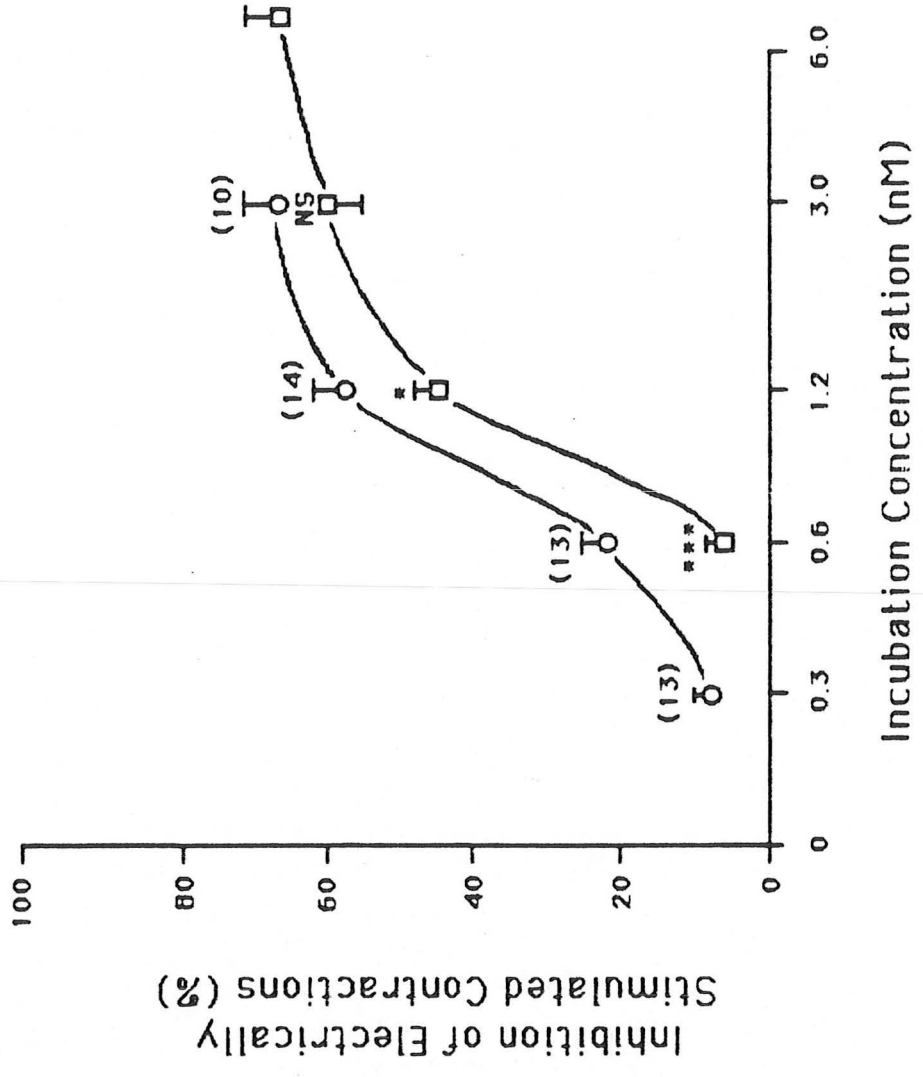

Inhibition of Electrically
Stimulated Contractions (%)
100
80
60
40
20
0
0
0.3
0.6
1.2
3.0
6.0
Incubation Concentration (nM)
(13)
(13)
(14)
(10)
***
*
NS

Table XXI. Acute Tolerance Effect Induced by DYN 1-13[1] on the GPI.

Time (min)	N	Inhibition of Electrically-Stimulated Contractions (%)[2]	Significance Relative to 0 time P
0	6	56.8 ± 1.8	-
30	6	48.1 ± 2.9	<0.05
60	6	42.5 ± 4.2	<0.01
100	6	39.9 ± 2.0	<0.001
130	6	30.6 ± 2.9	<0.001

[1]Concentration of DYN 1-13 used at all the times was 1.2 nM.

[2]Results are expressed as Mean ± S.E.

DISCUSSION

The observation that substituted aminotetralins have the potential for opioid agonist (Kandeel and Martin, 1973) as well as opioid antagonist activity has stimulated the synthesis and pharmacological evaluation of two new derivatives of 3-amino-2,2-dimethyltetralin (J and MRSAL). These compounds were studied for opioid agonist and antagonist capacity using the electrically-stimulated GPI and MVD preparations. Other investigators (Paton, 1957; Waterfield et al., 1977; Henderson et al., 1972) have demonstrated that the cholinergic junctions of the myenteric plexus of the GPI and the adrenergic transmission of the MVD are depressed by opioid agonists. The present study has confirmed that these isolated tissues are very sensitive to opioid actions and are very suitable for documenting the basic pharmacological properties of potential narcotic analgesics, such as their receptor affinity (potency) and their intrinsic activity (capacity for agonist or antagonist effects). It has been observed (Kosterlitz and Waterfield, 1975; Creese and Snyder, 1975) that, in general, there is a very good correlation between the relative agonist potency in GPI and the relative potency for analgesia in humans.

Since these isolated tissue preparations have multiple opioid receptors, it was also possible to study the opioid receptor specificity of J and MRSAL.

The results of these experiments demonstrate that J has actions that

qualitatively do not differ from those seen with other opioid agonists--J consistently caused a concentration-dependent decrease in contractions in GPI and MVD and these effects could be antagonized by NX.

On the other hand, MRSAL demonstrated significant opioid antagonist activity in a manner similar to that of NX. The antagonism of MRSAL on opioid receptors was specific, since it did not antagonize the inhibitory effects of epinephrine in GPI.

The agonist properties of J and its receptor specificity were studied by close examination of: (i) its relative potency to depress contractions in the GPI as compared to the MVD; (ii) its sensitivity to NX antagonism (analysis of Ke and pA_2 values) in both preparations; and (iii) its selective blockade of B-FNA activated mu receptors in GPI. Although these criteria are not completely satisfactory (mostly due to lack of selective ligands), they have been widely used and have led to the concept of multiple opioid receptors. The observation that the enkephalins are much more potent than morphine in the MVD but less potent than this alkaloid in the GPI has led Lord et al. (1977) to propose that enkephalins act on delta receptors in the MVD. Furthermore, the apparent dissociation constant (Ke value) of the opioid antagonist NX against mu agonists is very low (2-5 nM) compared to that against kappa agonists (20-40 nM) (See Tables XIII and XIV for references). The Ke value may not only be different in the same tissue (depending on which agonist is being blocked) but also different between tissues. For example, the enkephalins have high pA_2 values (Table XI) in the GPI suggesting interaction at mu sites in this preparation but lower values in the MVD (Table XV) suggesting that here they bind to delta receptors (Lord et al., 1977; Waterfield et al., 1977). Selective mu receptor alkylation

with B-FNA has demonstrated that normorphine, the enkephalins and fentanyl are mu receptor agonists while dynorphin and EKC are preferentially kappa agonists in the GPI (Huidobro-Toro et al., 1982) and MVD (Schulz et al., 1984). Alkylation of mu, kappa and delta receptors with B-CNA coupled with selective "protection" experiments have demonstrated that B-endorphin is selective for mu in the GPI but not selective for mu over delta and kappa in the MVD (Goldstein and James, 1984) and that dynorphin is kappa selective in guinea pig brain membranes (James et al., 1982a,b).

The antagonist properties of MRSAL were evaluated by its capacity to antagonize the decrease in contractions in GPI and MVD produced by prototype agonists of mu, kappa and delta opioid receptors. Its potency and selectivity for each of these agonists (Ke value) as well as its affinity for each type of opioid receptor (pA_2 value) were compared to that of NX.

The results obtained in these experiments demonstrated that all of the compounds tested showed some capacity to depress electrically-induced contractions in both preparations. All the reference opioids (NM, DHM, EKC, U-50,488H, B_H-END, DYN 1-13, Leu-ENK and DADLE) as well as the test opioid (J) behaved as agonists in that they all caused a concentration-related decrease in the contractions and that this effect could be antagonized by the nonselective opioid antagonist NX. In the present study, this reference opioid antagonist demonstrated poor agonist capacity causing a decrease in contractions of only about 7% (352 nM) and an increase in contractions of about 3% (704 nM) in GPI. This poor agonist property of NX has also been reported by Kajiwara et al., (1986). On the other hand, the test opioid antagonist, MRSAL, produced

an increase in muscular contractions at virtually all concentrations tested. MRSAL could produce a decrease in contractions in GPI only at high concentrations (850 μM). This latter effect could be indicative of an opioid agonist effect in this preparation but since no antagonism experiment was done with NX, it is not possible to specify the pharmacological receptor involved. Since such a high concentration was necessary to produce this effect, the experiment was not repeated. It is of interest to point out that this effect of decreasing contractions in GPI could also be due to the activation of alpha and/or beta adrenergic or dopaminergic receptors reflecting a non-opioid action. Such activity has been documented for several other aminotetralins (Cannon and Koble, 1980; Beaulieu et al., 1984; Cannon et al., 1980; DeMarinis et al., 1982). The increase in muscular contraction induced by MRSAL in the GPI is presumably due to an increase in release of acetylcholine by the postganglionic cholinergic nerves in the myenteric plexus. It has been well documented that opioid agonists inhibit release of acetylcholine in the GPI (Schaumann, 1957; Paton, 1957; Huidobro-Toro et al., 1981b) and in cerebral cortex (Jhamandas et al., 1977) and that this effect is specifically antagonized by NX. Furthermore, NX (30-100 nM) can cause an increase in the output of acetylcholine released by electrical field stimulation (0.017 Hz) in GPI (Waterfield and Kosterlitz, 1975) and rat cerebral cortex (Jhamandas et al., 1977). It has been proposed that this effect of NX is due to the blockade of endogenous enkephalins at the cholinergic terminals within the myenteric plexus in the ileum (Waterfield and Kosterlitz, 1975) and within the brain (Jhamandas et al., 1977). NX could also increase the output of acetylcholine by antagonizing other endogenous opioids such as dynorphin also present in the ileum. These

observations suggest that endogenous opioids physiologically control the release of acetylcholine from cholinergic neurons in the intestine.

Among all the opioid agonists tested, kappa prototypes (EKC, U-50,488H and DYN 1-13) demonstrated an extremely high potency in the GPI. The fact that the myenteric plexus of the GPI is very sensitive to kappa agonists is curious since there is as yet no information regarding kappa binding sites in this preparation or in the MVD. Except for DADLE, kappa drugs were also the most potent agonists in the MVD preparation. DADLE, a stable analog of Leu-ENK with reported delta receptor selectivity, demonstrated extraordinary potency in the MVD. This peptide is more potent than Leu-ENK in both preparations. The weak potency and rapid actions of enkephalins in vitro and in vivo have been attributed to their rapid degradation (Schwartz et al., 1981). Three types of enzymes (aminopeptidases, enkephalinase A and B) and also angiotensin-converting enzyme may account for the hydrolysis of enkephalins (Gorenstein and Snyder, 1979; Hudgin et al., 1981; Schwartz et al., 1981). All of these enzymes are found in brain tissue, and aminopeptidase activity is also present in plasma and in the GPI (Schwartz et al., 1981). The present study has shown that not only Leu-ENK, but also DYN 1-13 and B_H-END, spontaneously recover from inhibition to control contractions in different rates (Tables XVIII-XX). This spontaneous recovery of inhibition is assumed to be due to peptide degradation and not to tolerance at the receptor site since the preparation was still sensitive to subsequent Leu-ENK and B_H-END, but not DYN 1-13. The recovery rate to control contractions did not increase with the development of tolerance induced by DYN 1-13. The rate of spontaneous recovery varied among the three opioid peptides--Leu-ENK was the fastest (66%) whereas B_H-END was

the slowest (13%) at 5 min. The rate of spontaneous recovery is probably influenced by several factors such as: (i) the qualitative and quantitative distribution of the degradative enzymes in the tissue; (ii) the rate of dissociation of the peptide from its receptor, and (iii) the size of the peptide molecule. The slower rate of spontaneous recovery of B_H-END compared to that of Leu-ENK could have three possible explanations: (i) the enzymes responsible for peptide degradation present in the GPI are more specific for Leu-ENK than for B_H-END; (ii) the enzymes that degradate Leu-ENK are different from those that hydrolyze B_H-END, and (iii) the enzymes in the tissue produce active metabolites with independent opioid agonist activity. It should be noted that in the B_H-END molecule, the amino acid sequence 61-65 is Met-ENK, 61-76 is alpha-END and 61-77 is gamma-END and these all have opioid agonist properties (Rossier and Bloom, 1979). Sanchez-Blazquez et al. (1984a) reported a rate of spontaneous recovery of 59% for Leu-ENK and 51% for DYN 1-13 at 15 min. In the present study, a much faster rate of spontaneous recovery for both peptides was noted (see Tables XIX and XX). This difference could be due to the amount of protein/mg of tissue which would vary depending on the length of the strip used (3-cm or longer). We used a 3-cm long double strip forming a loop of 73.3 ± 1.6 mg (Mean ± S.E., N = 70). Slow and fast spontaneous recovery of contractions after B-END and the enkephalins, respectively, have also been described by Hughes (1975) and Cox et al. (1976). It appears that the rate of recovery occurs to differing extents in different ileum preparations.

Morphine-induced tolerance in the GPI was first described by Paton (1957) and confirmed by Gyang and Kosterlitz (1966). Recently, Wuster et

al. (1981a,b), Schulz et al. (1984) and Rezvani et al. (1983) have used the development of tolerance induced by opiates to assess different opioid receptor populations in the GPI and MVD. The phenomenon that DYN 1-13 induces acute tolerance in GPI has not yet been described in the literature. Tolerance induced by this peptide was time dependent but independent of the concentrations of the peptide used or sequence of administration to the bath. Tolerance is usually measured as a decrease in response to a given drug. This phenomenon is not well understood and can occur both in vitro and in vivo. Tolerance induced by DYN 1-13 may be due to a drug-induced change in conformation of the receptor to which this peptide normally attaches. This receptor may not be the same receptor of EKC and U-50,488H because in DYN-tolerant preparations there was no change in subsequent response to these opiates.

Pharmacological receptors have been traditionally identified and classified according to their relative potencies and their sensitivity to blockade by selective antagonists. Different potencies in different tissues by the same drug have established that the GPI has mainly mu receptors whereas the MVD has predominantly delta receptors. Potency of a compound is dependent on its affinity for a specific receptor site and on the number of specific receptors available in a tissue (Goldstein and James, 1984). Intrinsic activity of a compound depends on the relative capacity to activate or not activate the tissue once the drug has attached to its receptor. Both affinity and intrinsic activity are closely related to the chemical structures of the drug and its receptor. The type of receptor and its population clearly can vary between physiologically differentiated tissues, even when the same agonist is used.

Therefore, assuming that different potencies in different tissues are indicative of different receptor populations, one can conclude:

(i) Since the mu agonists NM, DHM and B_H-END are equipotent in the GPI and MVD, they are acting at the same receptor type in both preparations. This is also true for the kappa agonists EKC, U-50,488H and DYN 1-13. Furthermore, since compound J is reasonably equipotent in both preparations, this suggests that this agonist interacts with the same receptor in both tissues and lacks delta receptor activity in MVD. In general, all these agonists were slightly more potent in the GPI than in the MVD. This is probably explained by the receptor reserve (Goldstein and James 1984) in these two tissues. Quantitatively (Creese and Snyder, 1975; Leslie et al., 1980) and qualitatively (Lord et al., 1977; Waterfield et al., 1977), there are more mu receptors in the GPI than in the MVD. The MVD predominantly contains delta receptors. The kappa receptor population may be slightly less in the MVD than in GPI.

(ii) The very high potency of Leu-ENK in the MVD suggests that this peptide interacts with two different receptor populations in these two tissues. Leu-ENK and DADLE have greater affinity for the delta receptors present in the MVD than for the mu receptors present in the GPI.

Concentration-response curves give important information not only about the potency (affinity) but also about the intrinsic activity

(efficacy) of an agonist. Analysis of the regression lines in regard to slopes and maxima indicate differences between the agonists in the GPI and MVD. All the compounds tested behaved as strong agonists, but the opioid peptides, especially DADLE in the MVD, were the strongest agonists (slope = 60.5) demonstrating high affinity and intrinsic activity at the receptor site. Interestingly, NM was a more efficient agonist in the MVD (slope = 55.2) than in the GPI (slope = 38.6). All opioid agonists caused a near maximum inhibition (70-90%) in both tissues. The opioid peptides and synthetic alkaloids NM and DHM had the highest intrinsic activity. An interesting exception was Leu-ENK in the GPI. This lack of maximum response by Leu-ENK is probably due to rapid degradation of the peptide in this preparation, since its stable analog, DADLE, was able to produce 90% maximum response in GPI.

Despite its low potency, compound J was capable of producing a maximum response comparable to Leu-ENK and U-50,488H in the GPI and B_H-END and DYN 1-13 in the MVD.

The simple observation that a particular agonist is capable of producing a pharmacological effect does not give any information as to which receptor type is involved to produce that effect. Therefore, the use of highly selective antagonists is extremely important in receptor differentiation. Unfortunately, presently available opioid antagonists are only "sufficiently" selective. Although NX is more selective for mu receptors, it is highly unselective for kappa and delta sites. The relative affinity of NX for mu sites is 0.85, for kappa 0.06 and delta 0.09 in brain homogenates (Paterson et al., 1984). It is expected that a "selective" antagonist should have an affinity for a specific site at least 100 to 1000 times over its affinity for some other site. Despite

the fact that NX is not a selective antagonist, it has been widely used to characterize multiple opioid receptor selectivity. The use of antagonists to differentiate the opioid receptor selectivity of an agonist is based on the fundamental assumptions of classical receptor theory. If two agonists are acting at the same receptor site, they should have the same pA_2 value (or Ke) for a reference antagonist. In contrast, if the pA_2 value for a common antagonist is different for two different agonists, this means that these two agonists interact with different receptors (Arunlakshana and Schild, 1959). Therefore, the pA_2 value (or Ke) measures the affinity of the antagonist for the receptor of the test agonist.

The different pA_2 (or Ke) values for NX suggests that DYN 1-13 is an endogenous kappa ligand since the Ke value for NX against this peptide is similar to that against cyclazocine and EKC (Chavkin et al., 1982; Yoshimura et al., 1982a; Huidobro-Toro et al., 1982). However, this peptide demonstrates kappa agonist activity only in vitro and not in vivo (Lee, 1983).

The analysis of the pA_2 values for NX and MRSAL against all the reference opioids was not a simple task. Both compounds demonstrated different antagonistic potencies and affinities for each agonist and varied between the two tissues used.

In the GPI, mu agonists NM, DHM and the delta agonist Leu-ENK were all very sensitive to NX antagonism. The high pA_2 (and low Ke) value for Leu-ENK indicates that in this preparation this peptide activates mu receptors. A comparison of the pA_2 for NX against J indicates that J is an agonist at the same receptor occupied by DHM, B_H-END and Leu-ENK (all mu agonists in the GPI). This strongly suggests that J is active at mu

receptors. However, the small, but significant, difference between the pA_2 for NX against NM and DADLE *versus* J is unexplainable (Table XII), since these opioids are also *mu* agonists in the GPI.

A greater difference was seen with the *kappa versus mu* agonists making these experiments easier to analyze. EKC, U-50,488H and DYN 1-13 had pA_2 values for NX antagonism that were indistinguishable from each other suggesting that these agonists act at a common receptor. Since NX has lower affinity for *kappa* receptors, it is suggested that these agonists are acting at this site. The highly significant difference between NX pA_2 values against *kappa* agonists and J indicates that J is *not* acting on a *kappa* receptor in the GPI. J seems to fall in a class of "intermediate" pA_2 (NX) values like B_H-END and DADLE. It should be noted that higher pA_2 values for NX antagonism of B_H-END and DADLE have been reported (Table XIV).

In the MVD, the picture for pA_2 analysis was even more complicated. This is probably due to the presence of at least three opioid receptors (*delta*, *kappa* and *mu*) and the lack of specificity of agonists as well as antagonists. However, it was clear that NX had its highest affinity at DHM, NM (*mu*) and J receptor. There was no significant difference between NX pA_2 values against J and NM. Since this was not the case with DHM, it appears that J is a *mu* agonist like NM but not like DHM. It also seems that in this preparation, J falls in a class of compounds with "intermediate" pA_2 values. This suggests that J is primarily a *mu* receptor agonist but also may have some actions at *kappa* sites.

All the other reference opioid agonists were much less sensitive to NX antagonism in the MVD. This includes the *kappa* agonists (U-50,488H, DYN 1-13 and EKC) and *delta* agonists (DADLE and Leu-ENK) and B_H-END. In

this preparation, B_H-END is an agonist at mu and delta and possible kappa sites, whereas DADLE and Leu-ENK are specific delta agonists. Also, kappa agonists U-50,488H, DYN 1-13 and EKC act in the MVD in a manner similar to that in the GPI.

Discrimination of receptors based on NX antagonism (either from Ke or pA_2 values of NX) may provide misleading information concerning the receptor selectivity of an agonist due to the presence of multiple receptor populations in different tissues. It is well recognized that NX is not mu specific and also has affinity for kappa and delta (and epsilon and sigma) receptors in higher concentrations. In addition, the lack of receptor selectivity of most agonists further complicates the interpretation of Ke or pA_2 values of NX. Two illustrative examples are:

(i) Based on sensitivity of antagonism by NX, both EKC and DYN 1-13 are grouped together as kappa agonists. However, Wuster et al. (1981a,b) have demonstrated that DYN 1-13 displays greater cross-tolerance to MRZ 2549 (kappa agonist) than to EKC. This is probable because EKC also has strong mu affinity in addition to its kappa receptor affinity. In fact, Goodman and Pasternak (1984) have noted that EKC binds with high affinity to the mu_1 site, and unlabeled EKC is able to displace mu_2 (^{3}H-DHM) and delta (^{3}H-DADLE) with the same potency as [^{3}H]-EKC. This suggests that at least in brain tissue, EKC is capable of interacting with mu, kappa and maybe delta sites. Although these same experiments were not done in GPI and MVD tissues, it is possible that the same situation

occurs, since these tissues also have mu, kappa and delta receptors. A similarity between mu and delta binding sites in these isolated preparations and receptors in the brain has been observed (Leslie et al., 1980; Creese and Snyder, 1975). In the present study it was documented that EKC also has mu properties in the GPI, since its effects were significantly ($P < 0.01$, Fig. 11) antagonized by B-FNA treatment. Thus, EKC has mu and kappa properties in GPI and probably also in MVD, whereas U-50,488H and DYN 1-13 are better kappa agonists. However, all have indistinguishable pA_2 values for NX antagonism.

(ii) One way to illustrate that NX pA_2 value can be misleading is to characterize the receptor preference for B_H-END. In the GPI, the Ke value for NX antagonism against this peptide suggests an interaction at mu sites; however, in the MVD (a lower pA_2 value) it appears that this peptide is acting on receptors different from mu. In actuality, it has been shown that B_H-END is not selective for mu over delta or kappa in MVD by the use of alkylating experiments with B-CNA (Goldstein and James, 1984). In MVD, the pA_2 value for NX antagonism of B_H-END is lower than that for mu (therefore, B_H-END is not mu) and it is of the magnitude as those for delta and kappa agonists (see Table XV).

Furthermore, based on the pA_2 values of NX against morphine, ketocyclazocine and SKF-10,047, Su and co-workers (1981) concluded that the GPI also has sigma opioid receptors. These investigators further pointed out that the evidence for differentiation of kappa and sigma receptors is not as strong as the differentiation of these receptors from mu sites. Finally, Kajiwara et al. (1986) concluded that buprenorphine is an agonist on both mu and kappa receptors because the Ke value of NX against this opioid lies in between those against mu and those against kappa agonists.

All these observations suggest that pA_2 and Ke values for NX may predict whether a compound is preferentially a mu agonist by the simple demonstration of very high pA_2 and very low Ke values (high affinity). However, when the preparation has all three receptors sites, calculation of pA_2 values for NX may not explain the receptor selectivity of an agonist because the interpretation of the Ke value (Kosterlitz and Watt, 1968) is based on the assumption that an agonist acts on a single receptor type. Intermediate pA_2 and Ke values can only suggest that either the putative agonist is acting simultaneously at mu and kappa and/or delta receptors, or that the agonist is acting on a single, not-yet-identified receptor (Schulz et al., 1984). Arunlakshana and Schild (1959) recognized these complications and pointed out that "it is not known how far the principle of classifying agonists by their pA_x can be extended; it tends to break down . . . when applied to agonists with an indirect action."

In spite of this controversy, the pA_2 and Ke values for MRSAL antagonism against reference and test opioid agonists were calculated. Like NX, this antagonist showed different degrees of potency and affinity

for the different opioid receptors, but it is clearly not as potent an antagonist as NX. However, despite its low affinity, MRSAL was a full antagonist of opioid receptors. Its highest affinity was seen at mu receptors. In the GPI, the highest pA_2 and lowest Ke values for MRSAL antagonism was seen at mu receptors. The order of affinity and antagonistic potency in the GPI was Leu-ENK > DHM > NM > B_H-END > DADLE (all mu agonists in this preparation). On the other hand, MRSAL had lower affinity at kappa receptors, since lower pA_2 values were obtained for EKC and U-50,488H -- but not for DYN 1-13. Interestingly, this antagonist (in contrast to NX) demonstrated higher affinity for DYN 1-13 than for EKC and U-50,488H receptors. This low Ke value was not expected since DYN 1-13 behaves like a kappa agonist (Goldstein, 1984) in isolated tissues. This great difference in antagonistic potency (Ke values, Table XVII) against DYN 1-13 compared to that against EKC and U-50,488H may suggest that either DYN 1-13 has mu actions or is acting on a different receptor site from EKC and U-50,488H in the GPI. Since the pharmacology of DYN 1-13 does not completely agree with that predicted for true kappa agonists (Lee, 1983), it is possible that the receptor site for DYN 1-13 is not quite the same as that for the classic kappa drugs.

MRSAL had intermediate Ke and pA_2 values against J. This may indicate that while J preferentially has mu actions, it is also able to stimulate other receptors (kappa?). It could also indicate that MRSAL is simply not very selective for either mu or kappa receptors in the GPI.

In the MVD, MRSAL demonstrated lower antagonistic potency than in the GPI, but its highest affinity was also seen at mu sites. Highest Ke values were seen against DHM, NM and J. MRSAL's pA_2 values against these agonists were above 5.0, whereas those against kappa and delta prototypes

were lower. The kappa agonists had intermediate pA_2 and Ke values for MRSAL antagonism, whereas the delta prototypes had extremely low values (pA_2 4.05- 4.11).

The experiments with MRSAL suggest that this antagonist has its highest affinity at mu receptors, intermediate affinity when antagonizing kappa agonists and low affinity at delta sites.

MRSAL antagonism supports the argument that J is primarily a mu agonist, since this antagonist demonstrated comparable antagonist potencies against NM, DHM and J in the MVD. In the GPI, however, the antagonist capacity of MRSAL against J was lower than that expected for a mu agonist. Further delineation of receptor selectivity of MRSAL needs to be done using competitive displacement of selectively labeled opioids in GPI, MVD and brain membranes.

MRSAL has been tested in vivo for analgesic activity using the mouse tail-flick and phenylquinone-writhing tests by Dr. M. D. Aceto (NIH, 1985, personal communication). This compound was inactive at doses up to 30 mg/Kg in the tail-flick assay, but demonstrated analgesic activity in the writhing assay (ED_{50} = 21.9 mg/Kg; 6.8 - 70.8 = 95% confidence limits). It should be noted that this latter assay is more specific for the non-narcotic analgesics (aspirin, phenylbutizone, propoxyphene, etc.) than for the narcotic analgesics. As an antagonist of morphine in the mouse tail-flick assay MRSAL produced the following results: At a dose of 1 mg/Kg, MRSAL caused a 6% antagonism of morphine analgesia; at 3 mg/Kg, 10%; at 10 mg/Kg, 37% and at 30 mg/Kg, 43%. Higher concentrations were not used. These data indicate that MRSAL is a true pharmacologic antagonist of morphine analgesia and may block this alkaloid through some action on mu receptors.

To further delineate the receptor specificity involved in the agonist effects of J, B-FNA was used for the specific purpose of inactivating mu receptors. This technique should provide convincing evidence whether or not an agonist is selective for mu receptors. B-FNA has reversible agonist effects antagonized by NX with pA_2 values similar to those of mixed agonist-antagonists (nalorphine, pentazocine) and EKC. Therefore, Takemori et al. (1981) and Takemori and Portoghese (1985a) concluded that B-FNA is a reversible kappa agonist. As an antagonist, this agent has been reported to irreversibly antagonize morphine, NM, enkephalins and its analogs in the GPI and morphine, NM and other mu agonists in the MVD. However, Smith et al. (1984) have noted that B-FNA (10^{-8} and 10^{-7} M) blocks the effects of agonists on mu (morphine), kappa (MR-2033) and delta (DSLET, an enkephalin analog) receptors in the MVD.

In our experiments, B-FNA pretreatment irreversibly antagonized NM, DHM and J, while EKC was only partially blocked (Fig. 11). There was approximately a 90% blockade of the agonist effects of J in the GPI. These data further indicate that NM and DHM are acting on mu receptors and that J behaves like a mu agonist in GPI. Nevertheless, J may have a residual effect (10%) on either some mu receptors (spare receptors?) not inactivated by B-FNA or on kappa receptors. NX was used in low concentrations (176 nM) and it was able to antagonize the residual effect of J by 98%. This may indicate that some mu receptors were still available and J was able to interact with them. These B-FNA "insensitive" mu sites have been described by Takemori and Portoghese (1985b).

The fact that B-FNA also caused significant antagonism of EKC effects strongly supports the data of others that EKC also has mu actions in the GPI.

Finally, J has been shown to exert true morphine-like analgesic activity *in vivo*. In the mouse tail-flick assay, J was analgesic intraperitoneally (ED_{50} = 6.2 mg/Kg; 2.2-17.6 = 95% confidence limits) and almost equipotent to morphine sulfate (ED_{50} = 5.8 mg/Kg; 5.7-5.9 = 95% confidence limits). In the phenylquinone writhing test, this compound produced analgesia at 9.9 mg/Kg dose intraperitoneally. Surprisingly, J was inactive in the mouse hot-plate assay at doses of 5 and 20 mg/Kg. Like the tail-flick procedure, this assay is relatively specific for morphine-like analgesics. When tested using the Single Dose Substitution (SDS) procedure in morphine-dependent monkeys, J produced a linear dose-response related to a decrease in withdrawal signs. This procedure is considered to be highly specific for morphine-like activity. Doses ranged from 1.5 - 12 mg/Kg. However, J was not able to totally suppress the withdrawal syndrome (Dr. M. D. Aceto, NIH, 1985, personal communication). J at 30 mg/Kg intraperitoneally did not antagonize morphine in the mouse tail-flick assay. These data demonstrate that J is an analgesic agonist of comparable potency to that of morphine in the tail-flick assay and that it lacks significant opioid antagonist capability. The fact that J is able to suppress (at least in part) morphine withdrawal symptoms in SDS monkeys further supports the conclusion that J is primarily a *mu* agonist. *Kappa* agonists are analgesics but do not suppress morphine abstinence (Martin *et al.*, 1976). Taken all together, these data suggest that J is a *mu* agonist *in vitro* and *in vivo* and has narcotic analgesic properties similar to morphine.

Since there are no specific opioid antagonists, a very specific test to demonstrate that J is a *mu* agonist would be to demonstrate cross-

tolerance to morphine *in vitro* using the method of Rezvani *et al.* (1983). The principle of this method lies on the assumption that if two or more opioids exhibit complete cross-tolerance, they must be acting at identical receptors.

CONCLUSIONS

The purpose of this research was to characterize the opioid effects and the receptor selectivity of two new aminotetralin derivatives, J and MRSAL, using the electrically-driven GPI and MVD preparations.

Our studies demonstrated that J behaved as an opioid agonist in that it consistently depressed electrically-induced contractions in a concentration-dependent manner and its effects could be antagonized by NX. On the other hand, MRSAL demonstrated significant and specific opioid antagonist capacity in a manner similar to NX. This antagonist was effective whether it was pre-incubated or added after the reference opioid agonists (NM, DHM, EKC, U-50,488H, B_H-END, DYN 1-13, Leu-ENK and DADLE). Compound MRSAL was also able to antagonize its keto-analog, J.

The opioid receptor selectivity of these two aminotetralins was studied. Compound J seems to be an opioid agonist with mu receptor preference because: (i) J was equipotent in GPI and MVD and, therefore, did not act on delta sites in MVD; (ii) the effects of J were completely and reversibly antagonized by low concentrations of NX in both tissues; (iii) the agonist activity of J was irreversibly blocked by B-FNA pretreatment in GPI; and (iv) in antagonist experiments with its alcohol analog MRSAL, J was antagonized by MRSAL with comparable potency and affinity to that of NM and DHM.

The opioid antagonist, MRSAL, demonstrated its highest affinity for mu receptors and was able to effectively antagonize NM and DHM in both

GPI and MVD. Like NX, MRSAL appeared to be somewhat selective for mu over kappa receptors, but poor affinity was seen at delta sites in MVD. In contrast to NX, MRSAL demonstrated better antagonist potency for DYN-13 than for EKC and U-50,488H in GPI, suggesting that this peptide may act on a site different from that of EKC and U-50,488H.

The findings of the present study are important in that they relate the chemical structures of these compounds with their pharmacological effects. These aminotetralins demonstrated that a slight modification in the molecular structure of compound J (an agonist) resulted in a new molecule (MRSAL) now possessed with antagonist activity. Both compounds, the keto (J) as well as its alcohol analog (MRSAL), were able to interact with opioid receptors.

The manner by which synthetic and endogenous narcotic analgesics interact with opioid receptors is not well known, but based on the chemical structures of clinically active compounds, several opioid receptor models have been proposed (Beckett and Casy, 1954; Feinberg et al., 1976; Portoghese et al., 1981; Lee and Smith, 1980; Martin, 1984). The original model proposed by Beckett and Casy (1954) stated that the opioid receptor surface has three essential sites: (i) a flat area for the binding of the aromatic ring of the narcotic compound; (ii) an anionic site for interaction with the amine group; and (iii) a cavity. This model described only one binding site for the different opiate agonists. The idea of a single binding site allosterically modulated by sodium ions to an agonist or antagonist conformation has been advanced by Feinberg et al. (1976) and others have proposed the presence of two binding sites for interaction of morphine, enkephalins and beta-endorphin (Lee and Smith, 1980; Portoghese et al., 1981; Martin, 1984).

One common characteristic of all narcotic agonists and antagonists, including the aminotetralins, is the presence of a flat aromatic ring and an amine group in their molecular structure. The chemical requirements for the interaction of compounds J and MRSAL to opioid receptors are present in their molecular structures. The aromatic ring would "fit" the flat surface while the tertiary amine should bind to the anionic site of the opioid receptor. However, the dramatic change in pharmacological effects due to the relatively slight difference in structure between compound J and MRSAL cannot be explained easily. It has been well accepted that the introduction of an allyl or cyclopropylmethyl group on the amine of morphine-type agonists usually confers antagonist activity to the new compound (Feinberg et al., 1976; Casy and Parfitt, 1986). MRSAL, the alcohol analog of J, lacks both an allyl or cyclopropylmethyl group substitution. Since the most significant difference between J and MRSAL is the ketone versus alcohol function at C_1 position, our studies suggest that this modification may be very critical pharmacologically. It is possible that the interaction of MRSAL with the receptor can change the amine orientation or the manner of interaction of the aromatic ring to the receptor.

It would seem productive for chemists to modify further the structures of these compounds, since new molecules with greater affinity and specificity for opioid receptors may result.

As in the case of all unresolved molecules, there are multiple isomeric forms of J and MRSAL. Whereas with 3-(dimethylamino)-2,2-dimethyl-7-hydroxy-1-tetralol, there can be both configurational and optical isomers. MRSAL was comprised of a racemic mixture of the cis isomer. The other configurational isomer, the trans form (RMG), was

isolated as a racemate, and was also tested in the GPI preparation. In contrast to MRSAL, RMG showed agonist activity which was partially reversed by NX and MRSAL. RMG also exhibited poor antagonist activity towards NM, DHM and DADLE (Lippman et al., 1987). Therefore, the opioid antagonist activity of MRSAL was due to the cis configurational isomer rather than the trans isomer.

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