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Canada

THE UNIVERSITY OF ALBERTA

Investigation of twitch potentiation by opioids in frog's

skeletal muscle

by

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ABSTRACT

Morphine and several other opioid agonists including
the enkephalins caused a dual action on the twitch of the
isolated, curarised, and electrically stimulated frog toe
muscle; a potentiating action at low drug concentrations and
a potentiation followed by an inhibitory action at higher
concentrations.

The twitch potentiation was found to be
nonstereospecific and resistant to antagonism by naloxone.
The inhibitory action too was naloxone-resistant and is
probably due to a nonspecific local anesthetic effect of the
opioids on the electric properties of the frog skeletal
muscle fibre membrane.

An examination of the mechanism underlying the
twitch-potentiating effects of the opioids indicated that
the drug-effects were not due to recruitment of additional
muscle fibres or to a repetitive discharge in individual
muscle fibres. Thus, the responses observed with opioids
were not tetani but potentiated twitches.

Methadone decreased the critical fusion frequency of
the muscle and increased the time required by the twitch
response to attain its peak amplitude which suggested that
the drug prolonged the duration of the active state of the
muscle.

The rapid kinetics of the onset of the opioid effect
and of its reversal (following washout of the drug) coupled
with the sluggishness with which the compounds are known to

penetrate into the myoplasm suggested that their site of action is the membrane of the muscle fibre.

Experiments involving the use of K^+-induced contractures or of D-600 indicated equivocally that opioids do not promote the influx of Ca^{++} during depolarization of the muscle fibre.

The opioid-effects on the twitch were antagonised by increasing the concentration of the extracellular Ca^{++} from 1.08 mM to 8.64 mM.

Opioids did not displace the superficially bound Ca^{++} from the surface membrane sites of the muscle indicating that this is not the mechanism by which they cause twitch potentiations.

Low, twitch-potentiating concentrations of opioids did not alter the active electric properties of the muscle fibre membrane at a time when they potentiated the twitch suggesting that the opioid-effects on the twitch are not caused by changes in the electric properties of the membrane.

Experiments with quaternary naloxone suggested that opioids did not act on the outer or extracellular surface of the muscle fibre membrane to produce their effects on the twitch but acted presumably by dissolving in the membrane.

A study of the effects of opioids on the maximally summated muscle responses to closely spaced multiple electrical pulses revealed that an increase in the number of pulses in the pulse train causes a decrease in the percent

increase in the response height, in the presence of twitch potentiating concentrations of opioids. It is proposed that this effect is due to a depletion of the stores of 'trigger' Ca^{++} following stimulation with closely spaced multiple pulses.

The results suggest that opioids produce their effects on the twitch by facilitating the process of excitation-contraction coupling in the muscle. It is proposed that they do this by causing the release of additional amount of 'trigger' Ca^{++} following the electrical stimulus.

ACKNOWLEDGEMENTS

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1.   INTRODUCTION.

1.1 OPIOID RECEPTORS.

The psychological effects of the poppy were known to the ancient Sumerians who rather appropriately referred to the poppy as the 'joy plant.' The milky exudate from the flower seed capsule of the plant, later called opium, has been used in several countries down through the ages not only for its medicinal properties (e.g., pain relief, control of dysenteries, etc.) but also as a social drug that provided a sense of well-being, a dream-like state, and a calming effect. The word *opium* itself is derived from the Greek name for juice, the drug being obtained from the juice of the poppy, *'Papaver somniferum'*. By the middle of the sixteenth century, the uses of opium that are still valid were fairly well recognized, and in 1680, Sydenham (c.f. [1]) wrote:

> "Among the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium."

The modern era of opioid research can be said to have begun in 1803 when Seturner (c.f. [1]) isolated and described an opium alkaloid that he named morphine, after Morpheus, the Greek god of dreams. It was soon discovered that morphine was the major alkaloid responsible for most of the pharmacological and medicinal effects of opium. Since then the drug has opened a whole vista of research for innumerable scientists. The scientists were spurred not only by a desire to conquer pain but also by a desire to rid

society of the problems arising from the abuse of this compound.

1.1.1 Developments that led to the Receptor Postulate:

The problem of addiction to opioids stimulated a search for potent analgesics that would be free of the potential to produce addiction. Although this has not yet been achieved, thousands of compounds, more or less structurally related to morphine, were synthesized. A considerable amount of information on the structural requirements for analgesic action came out of this work. It was discovered that the analgesic action resides in only one of the enantiomers of a racemic mixture, usually the levorotatory isomer. Also, parts of the morphine molecule could be modified drastically or dispensed with entirely without causing major changes in pharmacological potency. On the other hand, even small changes in certain parts of the molecule resulted in profound effects on its pharmacology. For example, the substitution of the methyl group on the tertiary nitrogen of morphine by an allyl- or cyclopropylmethyl group, causes the resulting molecule to become a potent specific antagonist against many of the actions of morphine and related opioids. All these antagonists (e.g., naloxone, naltrexone, nalorphine, etc.) retain some of their 'agonist' properties [2, 3], i.e., they are partial agonists.

The stereoisomeric requirement and the structural constraints placed on analgesic actions of opioids [4 - 7] led to the 'stereospecific-receptor' hypothesis. This hypothesis postulated that opioids must bind to specific sites in the body and that this binding then triggers a sequence of events that result in the responses observed. These sites or receptors would permit only drugs with suitable structures and stereochemistry to bind.

1.1.2 Discovery of Opioid Receptors:

One of the earliest approaches to the investigation of the opioid receptor involved the comparative study of the analgesic effect of opioids in the whole animal. In this type of study, a systematic modification of the structure of a prototype drug was carried out and the potencies of congeners to produce a certain effect was compared. This allowed inferences to be drawn as to the receptor structure [4]. This approach has several limitations; since the observed nociceptive response is a consequence of a complex sequence of events initiated by the drug-receptor interaction, it may be modified by many factors. Also, the concentration of drug at the receptor site, which is affected by absorption, distribution, metabolism and excretion, is difficult to assess.

Opioid receptors were later discovered in an isolated peripheral tissue, viz., the guinea pig ileum. This

facilitated the investigation of the mode of action of opioids in a simple *in vitro* system. In this relatively simple model, the potency of opioids to inhibit indirectly stimulated contraction correlated well with their potency as analgesic agents; furthermore, their effects were competitively reversed by the narcotic antagonists naloxone and naltrexone [8 - 10].

Henderson *et al.*, [11] discovered opioid receptors in another peripheral tissue, viz., the mouse vas deferens. There was again an excellent correlation between inhibition of contraction and the analgesic potency of an opioid drug. Both tissues thus seem to contain receptors with specificities much like those postulated to exist in the brain.

A few years later, Frank [12, 13] reported the existence of opioid receptors in yet another peripheral tissue, viz., the frog sartorius muscle. He showed that the meperidine-induced block of action potential in the above muscle is produced by two mechanisms of action; one, a nonspecific local anesthetic-like effect, and the other, a specific inhibition of the gNa mediated by means of an opioid drug receptor associated with the muscle fibre membrane. Frank proposed that there were opioid receptors on the intracellular surface of the membrane associated with the Na$^+$ channels and that drug activation of these receptors interfered with the opening of the channels normally

produced by membrane depolarization. A later study [3] established the stereoisomeric requirement of the opioid-induced late occurring depression of gNa in frog skeletal muscle fibres. This served to reinforce earlier findings which demonstrated an interaction of opioids with opioid receptors in the frog muscle fibre membranes [12 - 14].

Hunter and Frank [15] demonstrated the presence of opioid receptors in yet another excitable membrane, the frog sciatic nerve axons. Analogous to Frank's earlier observations in frog skeletal muscles [12, 13], it was noted that in the sciatic nerve preparation too, meperidine depresses action potential production by two distinct mechanisms, viz., a non-specific local anesthetic effect and an opioid receptor mediated effect. The presence of opioid receptors on the surface membranes of neurones has also been reported by several other investigators [16 - 23]. In all these tissues it was observed that the depressant effects of opioids on the excitability of neurones could be antagonized by low concentrations of naloxone. These effects were also found to be stereospecific by those investigators who tested for such an attribute in the opioid action [16 - 18].

Early attempts at biochemical demonstration of the existence of specific binding sites for opioids were unsuccessful due to the difficulty of distinguishing between specific and nonspecific binding. In 1971, Goldstein and

co-workers [24] showed that, while [³H]levorphanol was bound stereospecifically to mouse brain homogenates, the stereospecific binding amounted to only 2% of the total binding. However, the use of low concentrations of labelled drugs with high specific activities allowed unequivocal demonstration of sites to which opioids such as dihydromorphine, etorphine and naloxone are bound in a saturable and specific manner [25 - 27]. In these experiments 50-90% of bound opioid was found to be bound stereospecifically (i.e., replaceable by unlabelled opioids but not by their inactive enantiomers).

A great deal of work was later devoted to characterization of the stereospecific binding sites and accumulation of evidence as to their identity with the postulated opioid receptors (See [28]). Binding assays provide information only about the interaction of opioids with the recognition site of the receptor. It is therefore necessary to attempt to correlate binding data with those from pharmacological assays. Excellent correlation has been seen in several laboratories between *in vivo* pharmacological potency and *in vitro* binding affinities of a large number of drugs that differ in analgesic potency over several orders of magnitude [29 - 31].

1.1.3 Distribution of Opioid Receptors:

Some of the earliest studies of receptors using radioactive binding techniques dealt with their regional distribution in the brain tissue of rodents, monkeys and humans [32, 33]. These regional distribution studies were of limited value because it is practically impossible to subdivide the brain into micro-size pieces to obtain a high resolution picture of the distribution of the 'receptors'. Thus, the development of a histochemical method to localize opioid receptors was the goal of very early studies. With regard to this goal, an important development was the ability to define conditions whereby one could inject opioids into the bloodstream of an animal and then find conditions where the bulk of the drug in the brain was localized to these specific receptors rather than nonspecifically distributed [34 - 36]. Once this was achieved it was possible to use a radioactive drug and to localize the receptor by autoradiographic methods with the light microscope. Using the technique developed by Stumpf and Roth [37] for autoradiographic localization of small diffusable molecules, several workers [35, 36, 38] were successful in mapping the opioid receptor.

A concerted effort was made by several workers to determine the distribution of opioid binding sites in the body. Biochemical and autoradiographic techniques were extensively employed to achieve this goal. These studies

were useful as they provided clues to the sites of opioid action.

Hiller et al., [33] embarked on a detailed study of the regional distribution of stereospecific [³H]etorphine binding using human brains obtained at autopsies. They studied binding in 39 anatomical regions of the brain. It was seen that the binding sites were not distributed uniformly, but there were large differences in levels ranging from 0.4 pmoles/mg protein in the olfactory trigone, amygdala and septal nuclei to virtually no binding in cerebral white matter, dentate nucleus of the cerebellum, tegmentum, and pineal and pituitary glands. The most interesting conclusion reached from this study was that most areas with high opioid binding were located in, or associated with, the limbic system. This system has often been suggested as a possible site of opioid action based on ablation and electrical stimulation experiments [39, 40]. The only area of the limbic system which did not exhibit high opioid binding was the hippocampus.

A similar study of the regional distribution of dihydromorphine binding was carried out by Kuhar et al., [32] in monkey brain with identical results. Atweh and Kuhar [38], using autoradiographic techniques showed 'opioid receptor' distribution at several levels of the spinal cord and lower medulla. This data showed a striking localization of binding sites to laminae I and II of the spinal cord, the

substantia gelatinosa of the spinal trigeminal nucleus, and in association with various nuclei associated with the vagus nerve. Also, administration of levallorphan, an opioid antagonist, blocked the autoradiographic grains and the accumulation of specific radioactivity in brain regions while administration of the inactive isomer dextrallorphan was without effect [35, 36].

These data are useful in understanding opioid drug action. For example, the substantia gelatinosa of the spinal cord is known to be strategically localized to modulate painful stimuli. High densities of opioid receptors at this level could, in part, account for the analgesic actions of opioids. Also, it is well-known that administration of opioids results in the suppression of visceral reflexes, such as the cough and the vomit reflexes. The high densities of opioid receptors in the vagus nerve and its nuclei could account for these particular effects since these visceral reflexes are mediated by the vagus system.

Are all of these binding sites opioid receptors? The locus coeruleus in the pons has a high density of opioid binding sites [36]. Immediately adjacent areas in the cerebellum have no detectable binding sites and other areas of the pons have a greatly reduced binding site density [36]. Accordingly, the effects of iontophoretic application of opioids on the firing rates of single cells in that area

were examined. Certain cells studied proved to be morphine sensitive, i.e., they responded promptly with cessation of spontaneous firing rate. These cells were also affected by naloxone in that it reversed the depression of the firing rate and blocked the depression when administered before the morphine. Also, most of those morphine sensitive units were localized to the locus coeruleus [41]. Many units not sensitive to morphine were found in adjacent areas. Thus, the distribution of morphine-sensitive units in this area of the brain corresponds to the distribution of opioid binding sites [41].

Such a correlation between opioid binding and opioid receptor mediated effect was not always found, e.g., the opioid agonist-antagonists, N-allylnormetazocine, pentazocine, and nalorphine are respectively 8, 34 and 12 times less potent in precipitating abstinence in the morphine dependent dog than they are in inhibiting the binding of the μ ligand [42]. On the other hand, methadone and fentanyl are respectively 11.5 and 278.5 times more potent in suppressing abstinence than inhibiting μ ligand binding [42]. The evidence obtained to date with the microscopic autoradiographic studies also do not always relate the ligand binding sites to known opioid pathways. The chemically-detected or autoradiographically-detected opioid binding sites when compared with immunocytochemical sites show some major unexplained discrepancies, e.g.,

though opioid binding sites are very dense in the caudate, it has very few immunoreactive fibres. Also, cerebral cortex, which has receptors if studied by binding shows sparse fibre or cell immunoreactivity (See [43]).

1.1.4 Discovery of Endogenous Opioid Ligands:

The discovery of opioid receptors and the finding that they existed in every vertebrate species tested triggered the active search for an endogenous opioid-like factor and ligand for the receptor. The rationale was the conviction that opioid receptors would not have survived evolution if their sole function was to bind to plant alkaloids. A physiological function that resulted in a selective advantage to the organism seemed probable. Such a function demanded the existence of an endogenous ligand, the binding of which was the real reason for the existence of these receptors. The search for such endogenous ligands began with a survey of known neurotransmitters and hormones, but none was found that could bind to the receptor with high affinity and also have opioid effects. Thus began a search for a new substance or substances with high affinity for the receptor and opioid-like actions.

The first reports of such endogenous opioid activity came simultaneously from two laboratories. John Hughes [44] in Hans Kosterlitz's laboratory at Aberdeen, Scotland, reported the presence of opioid activity in aqueous extracts

of animal brain, and Terenius and Wahlstrom [45] in Uppsala, Sweden reported the presence of a morphine-like substance in human cerebrospinal fluid. At about this time Teschemacher et al., [46] reported the presence of opioid activity in extracts of bovine pituitary glands. Hughes et al., [47] soon reported two pentapeptides that seemed to account for all the opioid activity present in extracts of pig brain. The pentapeptides were identified as Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu and were named by the authors methionine (met)- and leucine (leu)-enkephalin, respectively. The structures of the enkephalins were confirmed by Simantov and Snyder [48], who found and characterized peptides with the same structures, though present in a different ratio, in the bovine brain.

Another important observation made by Hughes et al., [47] was that the sequence of met-enkephalin was present in the structure of β-lipotropin (residues 61-65). β-lipotropin is a peptide hormone containing 91 amino acids that was first isolated by C. H. Li from pituitary glands in 1964 [49]. Soon, Ling et al., [50] isolated two peptides from extracts of sheep hypothalami and pituitaries. When these peptides were sequenced, they were found to represent sequences 61-76 and 61-77, respectively, of β lipotropin (β-LPH). At about the same time Cox et al., [51] and Bradbury et al., [52] independently found potent opioid activity in the C-terminal fragment (61-91) of β-LPH. These

longer peptides were named α (61-76)-, β (61-91)- and γ (61-77)- endorphin, respectively.

1.1.5 Multiple Opioid Receptors(?):

The discovery of opioid receptors and of endogenous opioid peptides, which are ligands of the receptors stimulated intense research in laboratories all over the world. Observations from several experiments led to the postulate that the opioid receptor existed in multiple forms. This conclusion had arisen from experiments conducted *in vitro* on intact tissues and *in vivo* with binding studies.

The concept of the opioid receptor subtypes was originally suggested by Martin [53] who observed that in man nalorphine had a dual action, antagonizing the analgesic effect of morphine and also acting as an analgesic in its own right. He concluded that the analgesic effect of nalorphine was mediated by a receptor which was different from the morphine receptor.

Martin and co-workers later described three different patterns of activities produced by morphine and its congeners in the chronic spinal dog [54, 55]. They proposed that these patterns indicate that opioid agonists interact at three distinct opioid receptor subtypes: μ, κ and σ for which the prototype agonists were morphine, ketazocine and N-allylnormetazocine (SKF 10047), respectively. Thus,

morphine induced analgesia, meiosis, bradycardia, hypothermia, and indifference to environmental stimuli. Ketazocine produced meiosis, general sedation, and depression of flexor reflexes but did not alter the skin twitch reflex or pulse rate. SKF 10047 caused mydriasis, tachypnoea, tachycardia and mania. Under this classification, opioids showed varying affinities towards each of the above receptor subtypes (Table 1).

Harris [56] evaluated the behavioral effects of several narcotic analgesics and the interactions between those narcotic analgesics and narcotic antagonists such as naloxone and naltrexone, by using schedule-controlled behavior. With this approach he was able to make distinctions between those drugs which were consistent with the three types of opioid receptors postulated by Martin and co-workers [54].

Subsequently, Lord et al., [57] and others [58 - 63] demonstrated evidence for the existence of heterogenous opioid receptor populations in several different tissues. In particular, studies involving competition of ligands for radiolabelled opioid binding sites in brain [57 - 61] and cross-protection studies involving inactivation of opioid binding by phenoxybenzamine and selective sulphydryl reagents [62, 63] have provided biochemical evidence for μ- and the putative δ-receptors; the latter are preferentially used by enkephalins. Wuster [64] proposed yet another

TABLE 1.

Varying affinities exhibited by opioids towards each of the putative opioid receptor subtypes.

Opioid	Receptor Subtype		
	μ	κ	σ
Morphine	High	Medium	Low
Ketazocine	Low	High	Low
Nalorphine	High	High	Low
Cyclazocine	High	High	High
SKF 10047	High	Medium	High

Note: Above matter was summarized from the work of Martin and co-workers [54, 55].

subtype of opioid receptor, the ϵ-receptor, to explain the high potency of β-endorphin in the rat vas deferens.

To reconcile the large amount of clinical and experimental data, many more sub-types of opioid receptors have been postulated. In fact, the total number of putative opioid receptors now number nearly a dozen and subspecies of μ, κ, and σ receptors have already been postulated (see [65]).

In spite of the voluminous circumstantial evidence for the presence of multiple opioid receptors, their existence has not been proven unequivocally. Recent work by several workers [66 - 72] has focussed attention on this problem. Zhang and Pasternak [67] used naloxazone, a long-acting (practically irreversible) opioid antagonist, and showed that the analgesic actions of morphine, D-Ala^2-Met^5-enkephalinamide, and β-endorphin were abolished in animals treated with this antagonist. When naloxazone was administered *in vivo* to rats and mice, it selectively inhibited for over 24 hr the high affinity binding of [^{3}H]morphine, [^{3}H]dihydromorphine, [^{3}H]naloxone, [^{3}H]D-Ala^2-Met^5-enkephalinamide and [^{3}H]ethyl ketazocine; in contrast, the 'low' affinity sites of these ligands were not affected by the drug treatment. Their results therefore suggest that all the ligands examined bind with highest affinity to the same population of opioid sites which may be called a receptor site because it is directly related to the

analgesic effect of the different agonists, μ, κ and δ. Therefore, they concluded that 'opiates' and 'opioid peptides' have a common analgesic mechanism mediated through a single high affinity binding site.

Several investigators are now thinking along similar lines, viz., in terms of a single or an interconvertible form of an opioid receptor. Pert and co-workers, who had earlier suggested the existence of a Type 1 GTP-sensitive opioid receptor and a Type 2 GTP-insensitive opioid receptor [73], hypothesized that the Type 1 opioid receptor is an interconvertible receptor which is able to adopt μ, δ and/or κ ligand selectivity pattern [69], and that the Type 2 opioid receptor is a receptor 'stuck' in a δ-like ligand selectivity pattern [70].

Lee and Smith [74] proposed the existence of a single universal β-endorphin receptor. Their concept of the 'multiple site β-endorphin receptor' stemmed from studies suggesting that β-endorphin interacted with both μ- and δ-receptor sites [57, 75]. β-endorphin is also active in all the *in vitro* systems, including the rat vas deferens, where most opioid alkaloids and natural enkephalin peptides have little, or no activity [57, 76]. The β-endorphin receptor was visualized to consist of two sites, one binding δ ligands and located possibly on a protein, and the other binding μ ligands and located on a lipid closely associated with the protein (Fig. 1). β-endorphin was proposed to

Membrane Surface
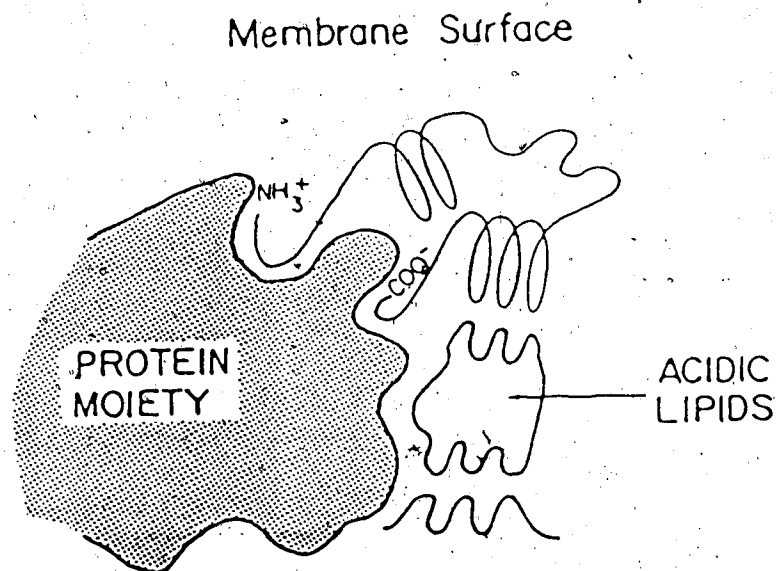
Figure 1. A protein-lipid model of the opioid receptor.
The proposed model of the opioid receptor consists of
both protein and lipid; the former contains a binding
site for enkephalins, and the latter a site for
alkaloids, with β-endorphin interacting with both sites.
[Lee, N. M. and Smith, A. P. (1980). Life Sci., 26:
1459-1464.]

interact with the δ-site through its N-terminal 'enkephalin' end, and with the μ-site through some portion of its C-terminal chain. This receptor could, under appropriate conditions, be activated by a ligand binding at either one of its sites, e.g., D-Ala^2-D-Leu^5-enkephalin at δ-site or morphine at μ-site to cause analgesia in the brain, or by β-endorphin, interacting with both sites (δ and μ) simultaneously [74]. Thus, this model predicts interaction between μ and δ sites under appropriate conditions since they both form a part of the same macromolecular complex. This prediction was borne out experimentally when it was shown that leu- and met-enkephalin, while analgesically inactive, could respectively, promote and inhibit, morphine-induced analgesia [77]. This model, however, could not deal with the existence of other possible opioid binding sites (viz., κ, ε, etc.). A similar two site receptor model has also been proposed by Rothman and Westfall [72] on the basis of binding studies with morphine and enkephalin.

Morley [78] proposed a 'common opiate receptor' model that he believes is consistent with all experimental observations thought to favour the 'multiple receptor' model. He believes that the properties of the common opioid receptor are a function of the environment in which they exist. Morley argues that the binding characteristics of ligands for the opioid receptor and the conformational changes that binding induces in the receptor may be altered

if the environment in which the receptor exists is altered.
Also, the altered environment would offer new opportunities
for additional, extra-receptor binding sites (or accessory
binding sites). The parts of the environment that are of
main concern are believed to be lipid molecules, membrane
proteins, and certain inorganic ions. Since the 'average
environment' would be different when a given preparation
(say, vas deferens) is derived from two different species
(e.g., mouse and rat), the binding characteristics of the
ligands for the receptor sites would be different. This
would then lead to the postulation of a new receptor type if
one believes in the 'multiple receptor' model. But
according to his 'multiple environment' model, an average
environment would lead to average pharmacokinetics depending
on the agonist/antagonist/radioligand being used.

Thus, according to the above model, tissues from which
a characteristic effect signal is generated, contain the
common opioid receptor in a characteristic average
environment, because of this environment, activation of the
receptor is only achieved with structurally distinct
agonists (presently classified as μ, δ, κ, or σ type
ligands). Also, this model predicts variation; an opioid
receptor in any specific tissue can change its ligand
characteristics to the extent that, at extremes of
environmental change, activation will cease to be achieved
by the particular type of ligand usually associated with it.

This model therefore accommodates β-endorphin as a ligand, differences in the distribution of μ- and δ-binding sites and ideas of κ, ϵ and other possible, opioid binding sites.

Thus, at present a controversy surrounds the issue as to whether μ, δ and κ opioid receptors are different entities or interconvertible forms of the same receptor. Results of selective tolerance and selective protection experiments would appear difficult to reconcile with an interconverting model of these sites. On the other hand, the rather similar distribution of the receptors in many areas of the brain argues for the 'interconvertible' hypothesis. It is also possible that the differences observed in the actions of various opioids are due to local tissue factors rather than multiple opioid receptors. The likelihood of such erroneous conclusions when dealing with receptors has been pointed out by Kenakin [79, 80]. Kenakin has attributed the existence of some receptor subtypes to the common misconception that tissue selectivity (of a drug) reflects receptor selectivity. Therefore the final resolution of the mystery of opioid receptor subtypes probably lies in the isolation, purification and sequencing of all the receptor forms.

1.1.6 Excitation Effector Coupling:

The binding of opioids to their 'receptors' has been extensively studied but information regarding the subsequent

events that ultimately result in the observed pharmacological response is as yet limited.

Cyclic 3', 5'-adenosine monophosphate (cAMP), which has been shown to be involved in the mediation of the effects of a number of hormones, has been suggested by Collier and Roy [81, 82] to play a role in the action of opioids. These authors have shown that the stimulation of cAMP formation by prostaglandins E_1 or E_2 in rat brain homogenate is inhibited by morphine and other opioids. There is no inhibition of the basal production of cAMP. This inhibition was seen at concentrations of opioids comparable to those required for analgesia and was antagonized by naloxone. The study of a series of opioids led to the finding that inhibition of prostaglandin-stimulated formation of cAMP correlated well with antinociceptive potency, opioid receptor binding affinity, and inhibition of electrically stimulated contraction of the isolated guinea pig ileum. Puri et al., [83] have reported, in apparent contradiction of the above results, that morphine produces a dose-dependent increase in the adenylate cyclase activity of rat corpus striatum. However, this effect is not stereospecific and is resistant to naloxone antagonism [84].

Certain cultured cell lines have large numbers of opioid receptors and therefore provide a relatively simple system for the study of postrecognition processes. In such cell lines, opioids inhibit basal as well as prostaglandin

E₁ stimulated adenylate cyclase activity in a stereospecific and naloxone-reversible manner [85 - 87], and this ability of opioid agonists to inhibit adenylate cyclase activity varies with the number of opioid binding sites present in the different cell lines [85].

Cyclic 3',5'-guanosine monophosphate (cGMP) levels in different regions of the brain too have been shown to be affected by opioids in a stereospecific and naloxone-reversible manner. In slices of rat striatum, leu-enkephalin, met-enkephalin, morphine, or levorphanol, but not dextrorphan, enhanced the accumulation of cGMP with a concomitant decrease in cAMP levels [88]. These effects were antagonized by naloxone. Regional differences in the effects of opioids on cAMP and cGMP contents have also been reported [89, 90]. Cyclazocine and naloxone ᴉcreased thalamic levels of cGMP, whereas cyclazocine caused an increase in the cerebellum.

While the interaction with adenylate cyclase may possibly be one of the initial events following opioid receptor activation, further biochemical changes may be of importance for the pharmacological action of opioids. In certain cultured cell lines, incubation for 24 hr with morphine, β-endorphin, and D-Ala², D-Leu⁵ enkephalin inhibited the biosynthesis of membrane glycosphingolipid and glycoproteins; these effects were stereospecific, dose-dependent, and naloxone-reversible and were not seen in

cell lines lacking opioid binding sites [91, 92].

Together with cAMP and cGMP, Ca^{++} constitutes the triad of 'second messengers' which control major biological events. Ca^{++} has often been proposed as an important factor mediating several of the pharmacological effects of opioids. One of the earliest biochemical observations which related the ability of Ca^{++} to antagonize the effects of opioids was reported by Takemori [93] who showed that morphine caused a decrease in the oxygen consumption of cerebral cortical slices at low Ca^{++} concentrations, but not in solutions with the usual Ca^{++} concentration. The analgesic effects of morphine were antagonized by intracisternal injections of Ca^{++} whereas they were potentiated by similar injections of ethylenediaminetetraacetic acid (EDTA) [94]. In fact, EDTA alone produced a weak analgesic effect which was diminished by equimolar doses of Ca^{++} [94]. The ability of Ca^{++} to antagonize opioid effects was enhanced by the ionophores A23187 or X537A [95, 96] which are known to increase the permeability of membranes to divalent cations. This suggests that Ca^{++} antagonizes opioid effects mostly at an intracellular site or sites.

Conversely, studies of interactions of opioids with Ca^{++} transport mechanisms in biological membranes have revealed a Ca^{++}-antagonistic effect of opioids. One of the earliest examinations of opioid effects on Ca^{++} fluxes revealed that morphine (10^{-3} M) inhibits K^+ (and EDTA)

stimulated ^{45}Ca influx and efflux in rat brain slices [97]. This effect was partially reversed by nalorphine. Later Kaku *et al.*, [98] reported that acute *in vivo* or *in vitro* morphine treatment caused a decreased ^{45}Ca uptake into mouse synaptosomal fractions. Similar studies using rabbit or rat synaptosomes showed that opioids reduce K^+-stimulated ^{45}Ca uptake in a naloxone-reversible fashion [99, 100]. That opioids affect Ca^{++} fluxes is also shown by studies dealing with Ca^{++} localization after subcellular fractionation of brain tissue which indicate that morphine produces a reliable and selective decrease in the Ca^{++} content of synaptosomal fractions [101 - 103].

Antagonism of opioid actions by Ca^{++} prompted investigators to examine the possible analgesic properties of La^{+++} and Ce^{+++}, agents which inhibit Ca^{++} binding and movement across biological membranes [104]. It was soon shown that lanthanides produce analgesia pharmacologically similar to opioid analgesia [105, 106]. The authors also showed that in animals tolerant to and dependent on morphine, La^{+++} suppresses withdrawal signs and the analgesic effect of La^{+++} is diminished. From experiments involving injections of morphine or La^{+++} into eight subcortical rat brain sites it was reported that these compounds appeared to share the same sites for antinociceptive activity [106]. The periaqueductal gray region of the midbrain was the most sensitive site examined

for both morphine and La^{+++}. This site has also been reported 'to possess the greatest opioid receptor concentration in the brain [32].

There are a number of reports suggesting that Ca^{++} is involved in the actions of opioids other than those mediating analgesia. Ca^{++} has been shown to antagonize the morphine depression of electrically stimulated ileum [107 - 109]. It has also been shown to antagonize the depression of neuromuscular transmission produced by morphine in the mouse vas deferens and the depression of synaptic transmission in the isolated sympathetic ganglia of rat and frog [110 - 112]. Using intracellular recording techniques, Rohani and Frank [113] showed that increasing the intracellular free Ca^{++} concentration in the frog sartorius muscle antagonizes the depressant effect on excitability produced by opioids acting on an intracellularly oriented opioid drug receptor.

Mule [114] demonstrated that opioids inhibited Ca^{++} binding to phospholipids in vitro. However, no correlation was observed when analgesic potency was compared with Ca^{++} binding inhibition. Thus naloxone and dextrorphan were more effective inhibitors than morphine, and it was concluded that effectiveness was related to the degree of drug ionization. Later, Mule [115] postulated an involvement at the membrane level between opioids, phospholipids and Ca^{++} based on the chemical similarity between the cationic charge

of Ca^{++} and the quaternary nitrogen of the opioid. He suggested that acidic phospholipids may serve as binding sites for these two positively charged species, and that opioids might compete with Ca^{++} for anionic binding sites on membrane phosphotides. Thus assuming that phospholipids act as receptors for opioids, the binding of opioids to phospholipids could lead to changes in membrane characteristics which in turn would produce opioid effects such as analgesia. It was reported that morphine inhibited ^{45}Ca binding to purified bovine gangliosides, while nalorphine exhibited a biphasic effect, enhancing binding at low concentrations and antagonizing binding at high concentrations. The above effect of morphine was partially antagonized by nalorphine [116].

In view of opioid effects on Ca^{++} movements, attention has been focussed on ATPase enzymes which serve to maintain active ion transport systems. Several experiments suggest that ATPase's may be important sites of opioid drug actions. Acute morphine treatment is reported to have caused increased ATPase activity (and increased Ca^{++} content) in adrenal cells, while chronic treatment caused a depression in enzyme activity (and decreased Ca^{++} content) [117]. The $Mg^{++}-$, $Ca^{++}-$ATPase activity of mouse brain synaptosomes was inhibited after acute morphine treatment [98]. Similar increases or decreases in ATPase activities following acute or chronic opioid treatment has been reported by several

workers [118 - 121]. Though a clear picture has yet to emerge from the differing effects of opioids on ATPase activities, these experiments do suggest that ATPase's might be involved in at least some of the pharmacological effects of opioids.

Thus, even though a voluminous literature exists detailing some of the biochemical events that follow opioid drug interaction with its receptors, the sequence of events following receptor stimulation and leading to the observed pharmacological response still remains a 'black box'.

1.2 CONTRACTION OF SKELETAL MUSCLE

"Muscle, which is the instrument of voluntary
movement ---- becomes thicker, shortens and gathers
itself together and so draws to itself and moves the
part to which it is attached".

Vesalius, 1543 (c.f. [122]).

Our understanding of the molecular events underlying
the contractile process has since become much clearer. The
investigation of the molecular biology of muscular
contraction was probably initiated by Kuhne in 1860 [123]
who extracted the contractile protein myosin, from muscle.
Later Edsall [124], and Muralt and Edsall [125] prepared it
in pure form and investigated its physical and chemical
properties. The discovery by Engelhardt and Lyubimowa [126]
that myosin prepared and purified by classical methods is a
specific ATPase revealed a link between the substrate of
contraction and ATP, a primary source of energy.

A major advancement in the field of muscle research was
the observation by Heilbrunn in 1940 [127] that Ca^{++}
activated the contractile machinery of the muscle fibre. He
proposed that this Ca^{++} was released from a cortical layer
near the surface of the muscle fibre following stimulation
of the muscle. At this time molecular biology of muscle
contraction was only at its beginning; Straub [128] had
recently discovered actin, and Szent-Gyorgyi [129, 130] and
Straub [128] were attempting to clarify the relationship of

actin to myosin and the effects of ATP on the proteins. In 1947, Heilbrunn and Wiercinski [131] showed that of all the physiological salts only Ca^{++} salts caused contraction of the muscle fibre when injected into it. These observations were subsequently confirmed by experiments in which Ca^{++} was introduced into frog muscle fibres by electrophoresis [132]. Experiments of this type and the very considerable amount of work on the activating effects of Ca^{++} on isolated muscle systems led to the idea that a release of Ca^{++} into the sarcoplasm is associated with contraction while its removal is associated with relaxation. At about this time, Marsh [133, 134] discovered a 'relaxation factor' in the muscle which afforded yet another major component of the contractile apparatus for *in vitro* experimentation. The availability of the main ingredients of the contractile apparatus in isolated forms, facilitated simulation of the contractile process in the test tube.

Today, it is a matter of common knowledge that the interaction between actin and myosin in the presence of ATP is the fundamental process underlying muscular contraction. In order to gain a deeper insight into the molecular events mediating muscular contraction, it is important to comprehend the ultrastructure of muscle and some properties of the associated muscle proteins.

1.2.1 Skeletal Muscle Structure:

Skeletal muscles are made up of thousands of individual muscle fibres, each of which is a multinucleated cell. Surrounding the muscle cell is a sheath, composed of glycoproteins (basement membrane material) and reticular fibres, and immediately under this is the muscle cell membrane also called the sarcolemma. Axial striations are visible under the microscope in skeletal muscle, and the repeat unit, the sarcomere, is bounded by highly refractile lines called Z-lines. Each muscle fibre contains several hundred to several thousand myofibrils, which are long, thin (1 to 2 μm in diameter) cylindrical structures lying parallel to the fibre axis. Around the myofibrils is the fluid sarcoplasm, and dispersed throughout it are the mitochondria, which in muscles are called sarcosomes.

The present concepts regarding myofibrillar composition, its organization and its function evolved from a pioneering paper by Hanson and Huxley in 1953 [135] in which they concluded that the two major contractile proteins, myosin and actin, are organized into two sets of parallel, interdigitating filaments. It is these interdigitating thick and thin filaments that give rise to the characteristic band pattern of skeletal muscle visible in the light microscope. The individual bands of the sarcomere were termed synonymously during the earlier part of the present century [136] but with the advent of electron

microscopy, the nomenclature has become more uniform. In polarized light the dark band is clearly birefringent and hence is called anisotropic (A band). It results from the aligned thick filaments. The light band is called isotropic (I band), although in reality it is slightly birefringent [137, 138]. The I band, which contains only thin filaments, is divided in half by a fine dark line, the Z line, which extends transversely across the fibrils. The Z lines are the attachment sites of the thin filaments. In the middle of the A band is a lighter region called the H band (for *hell*, the German word for light) which owes its existence to the thin filaments not meeting at the centre (Fig. 2). The thick filaments are bound together at their centres by cross connections, giving rise to a dark line, the M line [139].

X-ray diffraction and electron microscopic techniques have revealed the remarkable geometrical order of the contractile proteins of muscle. Vertebrate striated muscle examined in the wet state was seen to give rise to a strong and characteristic set of low-angle equatorial X-ray reflections [140]. These reflections arise from the hexagonal arrays of protein filaments which make up the contractile structure of the muscle. The relative intensities of the reflections indicated the existence of a double hexagonal array, in which myosin-containing filaments are situated at the lattice points and actin-containing filaments are situated at the trigonal positions
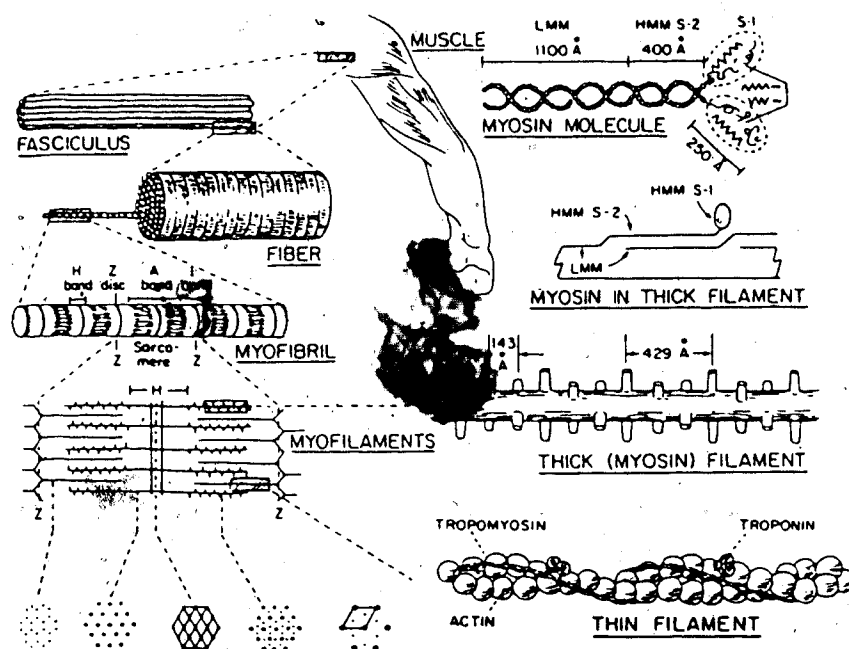
Figure 2. Skeletal muscle structure. Fine structure of
skeletal muscle. Schematic drawing of the structure at
different dimensional levels, from the whole muscle
through the muscle fibre, muscle fibril, filament, and
finally the molecular arrangement of the contractile
proteins. [Gordon, A. M., Physiology and Biophysics.
IV. Excitable Tissues and Reflex Control of Muscle.
Eds. Ruch, T. and Patton, H. D., W. B. Saunders Co.,
1982]

symmetrically between them (Fig. 2); this interpretation was substantiated by electron microscopy [140, 141].

Bailey, in 1948 [142], reported the existence of a new protein, tropomyosin, in the myosin fibril. That this protein is present in the I bands and the Z lines was reported by Perry and Corsi [143] who showed that proteins extracted from muscle fibrils using low ionic strength solutions consisted of tropomyosin and actin and that the fibril after such extraction showed no I bands or Z lines. Subsequently, it was shown that tropomyosin is an α-helical, rod-shaped molecule composed of two polypeptide chains in a coiled-coil configuration [144].

Ebashi and Kodayama [145, 146] discovered another myofibrillar protein which they called troponin. Later, Ohtsuki et al., [147] suggested, on the basis of antibody staining of isolated thin filaments, that troponin was distributed periodically at intervals of about 400 angstrom along the thin filament. This protein is actually a complex of three components, troponin C (TnC), troponin I (TnI), and troponin T (TnT) [148]. TnC is the Ca^{++}-binding component, and has four Ca^{++}-binding sites (Sites I - IV from the N-terminal of the polypeptide chain in sequence) [149]. Sites I and II have low affinity for Ca^{++}-binding, and sites III and IV have high affinity for Ca^{++} binding [149 - 151]. The high affinity Ca^{++}-binding sites have been reported to be able to bind Mg^{++} competitively [149]. Evidence to date

suggests that the Ca^{++}-specific sites are directly involved in the regulation of muscle contraction [152], while the high affinity sites may stabilize the troponin complex structure [153 - 157].

According to current models of muscle contraction, Ca^{++} binding to TnC produces structural changes that are propagated throughout other thin filaments proteins including TnT, TnI, tropomyosin and actin. These structural changes presumably alter the interaction of the troponin-tropomyosin complex with actin to allow the interaction of myosin with actin, ATPase activity and contraction [158 - 160].

TnT serves to bind whole troponin to tropomyosin [148] while the role of TnI is less clear. The latter has been shown to suppress actomyosin ATPase activity [161]. Structural changes have also been shown to occur in TnI with Ca^{++} binding or removal from the Ca^{++}-specific sites of TnC. These Ca^{++}-induced structural changes and their reversal are rapid enough to be directly involved in altering the TnI-actin interaction and activating muscle contraction [162].

Besides the major proteins discussed above, many others have been discovered in skeletal muscle: α-actinin, a protein isolated by Ebashi et al., in 1964 [163] is still in search of a function. A report by Stromer and Goll [164] suggests a possible function of this protein. They found

that after selective extraction of the Z-line material, the addition at 37°C of α-actinin results in cross-connections of thin filaments in the region, suggesting that α-actinin plays a role in the structure of the Z line. It was also found that this protein cross links F-actin strands at 0°C [165], suggesting that it might control the thin filament length. In contrast to the action of α-actinin, β-actinin, a protein first isolated by Maruyama [166], reduces interaction between F-actin molecules [167] implying a length determining role of the protein for thin filaments.

A protein preparation, antibodies to which stain the M line, was isolated by Masaki et al., [168]. Structural studies on the M line show two entities: M bridges attached to myosin and M filaments attached to M bridges [169]. They are believed to tie together the thick filaments and provide the enzyme creatine phosphokinase [139].

C protein is one of the contaminating proteins seen in myosin preparations and it has a strong affinity for myosin [170]. It was found to be distributed along the thick filaments with a spacing of 143 angstrom; [171] i.e. a spacing similar to the myosin cross-bridge repeat. Its role in muscle function is not yet clear.

Several other muscle proteins, e.g., paramyosin, a protein in invertebrate muscles [172]; parvalbumin, a Ca^{++} binding protein of striated muscle [173]; vimetin, a skeletal muscle protein [174]; filamen, an actin binding

protein in vertebrate skeletal muscle [175]; and H-protein and X-protein, components of the thick filaments of the vertebrate striated muscle [176] have been discovered but their roles in the functioning of muscle are yet to be elucidated.

1.2.2 Internal Membrane System

The present concepts regarding the internal membrane system of skeletal muscle evolved from two pioneering papers published in the 1950's. The first was an electron microscope study by Benett and Porter [177] showing the presence of a tubular network, the sarcoplasmic reticulum, in the interfibrillar space of skeletal muscle, and the second was a paper by Andersson-Cedergren [178] showing that the intracellular network of tubules in the muscle actually comprised of two distinct systems; one, the predominantly longitudinally oriented sarcoplasmic reticulum (or SR) and two, a predominantly transversely oriented tubular system (or T system).

The t-tubules are invaginations of the single membrane; this was first demonstrated in heart muscle [179]. The presence of such an opening could not be morphologically demonstrated for several years. Huxley [180], and Page [181] were the earliest researchers to demonstrate histochemically using electron microscopy that the T system of frog skeletal muscle communicates with the extracellular

space and that this sytem is distinct from the SR. Later, however, several investigators have demonstrated the existence of such an opening [182]. The t-tubules leave the membrane at different angles with regards to the fibre surface. In most cases their course is more or less perpendicular to the surface, though there are exceptions. For instance, some of them have been shown to run longitudinally [180], although such an occurrence may only be about 3% of the total T system. With electron microscopy, it was shown that the T system follows a spiral route with a very small slope around the axis of the whole fibre [183].

The excitability of the T system has received considerable attention in the past few decades. Numerous experiments published in the last 25 years corroborate Huxley and Taylor's [184] early observation that tubular depolarization rather than surface membrane depolarization was responsible for muscle contraction [185]. The fact that the T system is not merely a channel conducting the depolarization of the surface membrane electrotonically into the interior of a muscle fibre, but that its membrane can produce regenerative conduction was clearly shown by several investigators [186 - 189]. Also, the excitability of the T system was shown to be necessary for physiological contraction [190 - 193]. That the T system has qualitatively similar electrophysiological properties as the

surface membrane was further shown when Adrian *et al.*, [194] reported the presence of a delayed rectification in this system.

The SR of skeletal muscles consists of three morphologically and functionally distinct regions: the junctional SR that forms the triad junctions with the t-tubules; the terminal cisternae with an electron dense content; and the longitudinal elements of SR, which are slender tubules that connect the cisternae through the centre of the sarcomere and the Z line. The membrane envelopes of the terminal, cisternae and the longitudinal tubules are rich in Ca^{++}-transport ATPase. In the vicinity of the triad, the structure of SR changes. The clusters of Ca^{++}-transport ATPase molecules are replaced by larger and less densely packed particles, which are characterisitic of the junctional membrane and are presumed to participate in the transmission of excitatory stimulus from the t-tubules to the SR [195].

Membrane preparations of SR obtained from muscle homogenates by differential centrifugation contain microsomes derived from all three regions of the SR, contaminated with t-tubule and surface membrane elements [196, 197]. The crude microsome preparations can be resolved into t-tubule, terminal cisterna (heavy microsome), and longitudinal tubule (light microsome) fractions [197 - 201]. The isolated transverse tubules are characterized by

a high cholesterol content, the presence of Ca^{++}, Mg^{++}-activated and Na^+/K^+-activated ATPase, β-adrenergic receptors, and isoproterenol stimulated adenylate cyclase activity [202, 200, 203, 204]. Besides the Ca^{++}-ATPase, the SR contains a proteolipid with an apparent molecular weight of 12,000 [205]. Its function is unknown. Calsequestrin, a Ca^{++}-binding glycoprotein [206] has been shown to be present in the heavy microsome fraction [207]. In view of its Ca^{++}-binding capacity, calsequestrin was suggested to participate in the binding of accumulated Ca^{++} within the SR [207]. In addition to the above mentioned proteins, the SR has been shown to contain a high-affinity Ca^{++}-binding protein and a glycoprotein [208]. Their functions are unknown.

Lipids account for 45% of the total mass of lyophilized SR vesicles. About 90% of the lipid fraction is a mixture of phospholipids. Phosphatidylcholine accounts for 50-70% of the phospholipids. The remainder includes phosphatidylethanolamine, sphingomyelin, phosphatidylserine, and phosphatidylinositol. Neutral lipids include cholesterol, cholesterol esters, triacylglycerols, and free fatty acids. There is a large body of evidence showing that lipids are of fundamental importance in maintaining the function and the structure of the $Ca^{++} + Mg^{++}$-dependent ATPase [209].

Following the pioneering work of Ebashi and Lipmann [210], it has been firmly established that the ATPase of the SR can use the chemical energy derived from the hydrolysis of ATP to build up a transmembrane Ca^{++} concentration gradient. For each molecule of ATP hydrolysed, 2 Ca^{++} are transported across the membrane [211, 212]. The probable reaction sequence of Ca^{++} translocation has been described by de Meis and Vianna [213]. According to their sequence, interaction of 1 Ca^{++} and 1 ATP with the enzyme is followed by the phosphorylation of the enzyme, the transfer of Ca^{++} across the membrane, and its eventual release on the internal membrane surface. A Mg^{++}-dependent hydrolysis of the phosphoenzyme releases inorganic phosphate on the external membrane surface. The cycle is completed by the isomerization of the enzyme. The process is reversible [213, 214] and permits the synthesis of 1 molecule of ATP for each 2 Ca^{++} released across the membrane. Thus only one molecule of ATP is broken down for each 2 Ca^{++} transported.

The rate of Ca^{++} uptake by SR probably accounts for the rate of muscle relaxation. The physiological role of the observed reversal of the Ca^{++} pump is less clear. While it may significantly contribute to the release of activating Ca^{++} during muscle contraction, the speed of the activation process prompts the consideration of other mechanisms (gated channel etc.) as well [215].

1.2.3 Excitation - Contraction Coupling:

The contractile activity of muscle is regulated by the cytoplasmic free Ca^{++} concentration [216]. In resting fast-twitch skeletal muscle much of the intracellular Ca^{++} is sequestered within the sarcoplasmic reticulum and the cytoplasmic free Ca^{++} concentration is in the range of 10^{-7} - 10^{-8} M [217]. Contractile activation in skeletal muscle fibres is triggered by depolarization of the fibre surface membrane. Depolarization spreads inwardly along the membranes of the t-tubules and triggers the release of Ca^{++} from intracellular storage sites, viz., the terminal cisternae of the SR [218]; the cytoplasmic Ca^{++} concentration increases, and Ca^{++} binding to troponin initiates the complex set of reactions that lead to actin-myosin interaction and shortening as discussed in the preceding text. The entire sequence of events, initiated by electrical depolarization of the plasma membrane and culminating in mechanical activation of the contractile myofibrils within the membrane, has been termed 'excitation-contraction coupling' by Sandow [219]. Although a general consensus exists as regards the molecular basis of most of the events in the excitation-contraction (E-C) coupling process, a vital link, viz., the process whereby depolarization of the t-tubule causes release of Ca^{++} from the SR (T-SR coupling), is still a matter of debate.

Several theories attempting to resolve this mystery have been put forward but the one that has withstood the test of time is the 'trigger Ca^{++} hypothesis' for E-C coupling in skeletal muscle [220 - 226]. According to this hypothesis there are Ca^{++} bound to the intracellular surface of the t-tubular membrane which are referred to as the 'trigger' Ca^{++}. Some of these 'trigger' Ca^{++} can be released into the triadic junction by depolarization, produced either by action potentials or by some drugs (e.g., acetylcholine). The Ca^{++} diffuse across the triadic junctional space to the terminal cisternae of the SR where they stimulate the release of much larger amounts of Ca^{++} into the myoplasm. This raises the free intracellular Ca^{++} concentration sufficiently to stimulate the contractile proteins and initiate a mechanical response. The large increase in free Ca^{++} concentration also replenishes the 'trigger' Ca^{++} stores and stimulates active Ca pumps on the SR and on the surface membrane of the fibre. The latter effects lead to a rapid restoration of the intracellular free Ca^{++} concentration to a low level which terminates the response.

It also is postulated that there are superficial binding sites on the luminal or extracellular surface of the t-tubular membranes which are normally occupied by Ca^{++}. Occupation of these extracellular sites by Ca^{++} tends to strengthen the binding of the 'trigger' Ca^{++} to the

t-tubular membrane so that when the extracellular Ca^{++} concentration is reduced, the binding strength is reduced and more 'trigger' Ca^{++} is released into the triadic junction by an action potential and the twitch is potentiated. When the extracellular Ca^{++} concentration is raised the reverse happens.

Several other hypothetical coupling mechanisms have been proposed to explain the E-C coupling process in skeletal muscle. Most of these theories ascribe 'coupling' roles to the junctional feet and particles that have been observed by electron microscopy in the junctional gap of triads of skeletal muscle [227, 228]. One theory proposes direct flow of ionic current between the T and SR lumina through conducting pores in the feet. Tubular depolarization directly leads to electrotonic spread of potential across the triad to the SR, where depolarization can activate a voltage-dependent Ca^{++} permeability. So far, the clearest evidence from several experimental approaches has been contrary to the existence of free communication through a pore across the triad [229, 218]. Hodgkin and Huxley [230] proposed a basic mechanism for sensing and responding to a change in membrane potential. They postulated the existence of charged or dipolar molecules that changed position or orientation within the membrane as a result of changes in the membrane electric field. Subsequently, charge displacement currents, attributed to

the movement of intramembrane charges were identified in muscle [231]. This charge movement was tentatively associated with depolarization-contraction coupling. The charged group in the t-tubule was postulated to be linked mechanically to a plug blocking one SR Ca^{++} channel, with charge movement pulling the plug and unblocking the channel [232]. The similarity of number of charged groups detected electrically and of 'feet' between t-tubules and SR determined by electron microscopy provided the basis for assigning one T-SR link to each charged group [231]. One experiment that seemed to provide rather strong evidence that charge movement may not be involved in Ca^{++} release was the finding that when a fibre is highly stretched, the charge movement goes away, although Ca^{++} release seems to remain [233].

Franzini and Nunzi [234] have examined the junctional feet of triads in fish muscle and have presented evidence indicating an actual continuity between the feet and components of the t-tubular and SR membranes. Their more detailed description of the triadic junction does not answer how the junction operates. Continuity between feet and the two membranes does not exclude the possibility that the only function of the feet may be to provide a mechanical coupling between the membranes. This is needed to maintain the membranes at a fixed distance despite the distortions due to shortening and widening of the fibre during contractions.

If the message passing from the t-tubules to the SR during excitation is of a chemical nature, say 'trigger' Ca⁺⁺ [226] then the maintenance of a uniform separation between the two membranes and thus a mechanical role of the feet, are important. Thus, Oota and Nagai [235] reported that treating frog's skeletal muscle with hypertonic urea containing solutions followed by return to normal Ringer's solution disrupted E-C coupling without blocking the openings of the t-tubules at the surface of the muscle fibres. They attributed the block of E-C coupling to an increase in the width of the triadic junction produced by the urea removal treatment. Their findings and conclusions were confirmed and supported by Frank and Treffers [236].

Thus, from the various hypotheses proposed to explain the process of E-C coupling in skeletal muscle, the 'trigger Ca⁺⁺ hypothesis' seems most plausible. On the basis of this hypothesis, several actions of multivalent cations on contractions or contractures could easily be explained [222, 237]. This theory has also received support from the observation that Ca⁺⁺ applied to the cytoplasmic surface of the SR can induce a release of Ca⁺⁺ from the SR under appropriate conditions [238, 239]. Although, the physiological significance of this phenomenon has been questioned [240], the most recent evidence appears to support a physiological role for this effect [241, 242]. The other mechanisms proposed for E-C coupling cannot

incorporate several experimental observations [226, 243] and therefore cannot be considered as more than interesting speculations.

1.2.4 Role of Action Potential in Muscle Contraction:

The action potential precedes the twitch by a definite time interval (about 2 msec). It generates a process that runs its course during the latent period, and perhaps also later, and then activates contaction. To evaluate the role of the action potential in E-C coupling it would be necessary to determine the quantitative relation between membrane potential and tension output under a variety of physiological conditions, but this cannot be directly established for the twitch in terms of the potentials produced by the action potential itself since these continuously and rapidly change with time. Hodgkin and Horowicz [244] circumvented this problem by studying instead depolarization-induced contractures in single fibres of frog skeletal muscle by abruptly exposing them to solutions containing increased concentrations of K^+. Their results indicated the following: a) Tension output begins to appear only when the membrane is depolarized to a threshold value of about -50 mV, i.e., to the mechanical threshold; b) As the membrane is depolarized beyond the mechanical threshold, the tension-potential relationship rises in a steeply sloping S-shaped curve; and c) the tension output appears

to be maximal, i.e., saturated, at a membrane potential around -20 mV.

Sandow and co-workers [245] applied the above results to the problem of relating changes of membrane potential due to the action potential to production of mechanical change. However, in doing so they assumed that the action potential becomes capable of producing mechanical activity as soon as it depolarizes the membrane to mechanical threshold, and that it continues to be mechanically effective as long as it maintains the membrane potential beyond threshold. The time interval during which the spike maintains the membrane beyond mechanical threshold was termed the mechanically effective period (MEP). They presented data presumed to show that the action potential not only elicits contraction but also regulates the detailed form of the contractile response. The results were obtained using various agents that simultaneously affected both the mechanically effective period of the action-potential and the twitch mechanical output. They concluded that the role of the action potential in E-C coupling is to swing the membrane potential beyond the prevailing mechanical threshold for the duration of the mechanically effective period and thus initiate the sequence of coupling reactions activating contraction. They proposed that during the MEP the action potential continuously develops the condition activating contraction in the myofilaments, and that this condition is more

effective in causing contraction the lower the mechanical threshold and the longer the action potential duration. Thus, according to them an enhanced twitch response is produced by an agent that decreases the mechanical threshold (Type A agents, e.g., NO_3^-, caffeine) and/or increases the duration of the action potential (Type B agents, e.g., Zn^{++}, UO_2^{++})

Although Sandow and his co-workers [245] strongly ascribe to the above theory, several other researchers have expressed doubts about its validity. Kao and Stanfield [246] studied the effects of several ions on the electric properties of the frog sartorius muscle and its mechanical threshold. Their findings indicated that there is no direct causal relation between delayed rectification and mechanical activation. Mashima and Washio [247] observed that the spike duration and the twitch tension increased progressively with progressive increases in Zn^{++} concentration up to an optimal value (50 μm), any further increase in Zn^{++} concentration caused a decrease in twitch tension even though the spike duration continued to increase. This clearly showed that the two were unrelated. A similar effect was also observed by Sandow's group [248] but they argued that high Zn^{++} concentrations (50 - 1000 μm) raise the mechanical threshold of E-C coupling and reduce the intrinsic strength of the contractile system and therefore the twitch tension output gets smaller even though

51

the spike duration increases. The basic assumption made by
Sandow's group in the use of K^+-induced contracture as a
model for events occurring in an action potential-induced
twitch, is that depolarization in both cases initiates an
identical series of events culminating in the respective
responses. But we now know that this is not true; twitches
utilize membrane-bound Ca^{++} whereas K^+-contractures utilize
extracellular Ca^{++} in the manifestation of their respective
responses [243]. Therefore the mechanical thresholds for
depolarization contractures may not represent the mechanical
thresholds for action potentials. Therefore it would be
incorrect to ascribe to the twitch definite values of the
mechanical threshold derived from contracture experiments.

Thus, in view of the above mentioned discrepancies and
the incorrect assumptions made by investigators in this
field, the postulated role of the action potential duration
or area in regulating the twitch size is suspect.

2. STATEMENT OF THE PROBLEM.

The existence of opioid receptors in the excitable membrane of frog's skeletal muscle fibre has been reported by several invesigators [3, 12, 13, 14]. Also, it was reported that meperidine, an opioid agonist potentiates twitches in the directly stimulated frog sartorius muscle [249]. In view of the above reports, it was of interest to see if the two were related. The object of this thesis was therefore to investigate if opioids, in general, produce a twitch potentiating effect in the frog's toe muscle and if so, to investigate the possible involvement of a stereospecific opioid receptor in the response, employing classical organ bath techniques. Another objective was to investigate the basic mechanism underlying the above phenomenon of twitch potentiation by opioids in order to improve our understanding of opioid-effects on skeletal muscle.

3. MATERIALS AND METHODS

3.1 MATERIALS:

3.1.1 Solutions:

Ringer's solution prepared in distilled/deionized water was used as the physiologic solution in all experiments. The composition of the bicarbonate-buffered Ringer's solution (normal Ringer) was (in mM/liter): NaCl, 111.8; KCl, 2.47; $CaCl_2$, 1.08; $NaHCO_3$, 2.38; NaH_2PO_4, 0.087; dextrose 11.1; and *d-tubocurarine* 0.1 mg/ml. Tubocurarine was employed to eliminate possible neuromuscular effects.

The composition of the Ringer's solution was at times altered to suit the specific requirements of certain experiments:

- The Ringer's solution for some experiments was prepared without Na^+ or with a reduced Na^+ content and in either case choline chloride was substituted for NaCl in appropriate amounts to maintain osmolarity (230 mosmol/Kg.)

- In experiments involving the use of La^{+++}, normal Ringer's solution was not used as the physiologic buffer because the bicarbonate and phosphate ions present precipitate with the trivalent cation. Therefore in such experiments, a Tris buffer was used in the Ringer's solution. The Tris buffered Ringer's solution (Tris Ringer) contained 2 mM/liter of Tris(hydroxymethyl) aminomethone (Tris) HCl in place of

NaHCO$_3$ and NaH$_2$PO$_4$ in the normal Ringer's solution.

- In experiments requiring the use of Ca^{++}-free physiologic solution, CaCl$_2$ was omitted from the Ringer's (normal or Tris) solution and no attempt was made to chelate or otherwise exclude Ca^{++} present as trace contaminants of the other substances in the solution. It was previously reported that solutions prepared in this way contained less than 10 ± 1.3 x 10^{-*} M Ca^{++} [250].

- In experiments involving the use of a 25 mM K$^+$ solution, the required amount of KCl was added to the Ringer's solution without reducing other ions.

All solutions were prepared fresh for each experiment. The osmolarity of the solution was 230 mosmol/Kg and the pH was adjusted between 7.2 and 7.4.

3.1.2 Drugs:

Drugs used in this study were:

(a) (±)Morphine hydrochloride - May & Baker, Mississauga, Ontario, Canada.

(b) Methadone hydrochloride - May & Baker, Mississauga, Ontario, Canada.

(c) Codeine hydrochloride - May & Baker, Mississauga, Ontario, Canada.

(d) (+) Morphine hydrochloride ' - Dr. K. C. Rice,

' The compound was obtained through the kind courtesy of Dr.

National Institutes of Health (NIH), U.S.A.

(e) Fentanyl Citrate - McNeil Labs

(f) Etorphine hydrochloride -Reckitt & Colman ,

(g) (-)Ketazocine' - Sterling Winthrop

(h) (-)Ethyl Ketazocine' - Sterling Winthrop

(i) Met-enkephalin - Peninsula Lab. Inc. or Sigma
Chemical Company

(j) Leu-enkephalin - Peninsula Lab. Inc. or Sigma
Chemical Company .

(k) Levorphanol tartrate - Hoffman-LaRoche

(l) Dextrorphan tartrate - Hoffman-La Roche

(m) Naloxone hydrochloride - Endo Labs. Inc.

(n) Quaternary naloxone' - Boehringer Engelheim.

(o) Naltrexone hydrochloride - Endo Labs. Inc.

(p) Mr2096' - Boehringer Engelheim

(q) Meperidine hydrochloride - Winthrop Labs, Inc.,
Aurora, Canada.

(r) Procaine hydrochloride - Matheson Coleman & Bell

(s) Hyoscine hydrobromide - Penick Canada Ltd., Canada.

(t) Phenoxybenzamine - Smith, Kline & French

(u) Caffeine - Nutritional Biochemicals Corp.,
Cleveland, Ohio.

(v) A23187 - Sigma Chemical Co., St. Louis, Missouri.

(w) D-600 hydrochloride - Knoll AG, Ludwigshafen.

'(cont'd) K. Ramabadran from the respective sources.

Drug solutions were routinely prepared by dissolving the required amount of the substance directly in Ringer's solution. Exceptions to the general procedure were as follows:

- Solutions of phenoxybenzamine, (+)morphine, (-)ketazocine, or (-)ethyl ketazocine were prepared by initially dissolving a weighed quantity of the substance in minimum quantity of concentrated HCl solution (<20 μL) and then diluting with Ringer's solution.

- In the case of met-enkephalin and leu-enkephalin, stock solutions of the compounds (0.5 mg/3 Ml and 5 mg/3 Ml, respectively) were initially prepared in distilled water and stored in a frozen condition. Whenever required, the stocks were thawed, samples removed and appropriately diluted with Ringer's solution.

- Caffeine solution was prepared by dissolving the requisite amount of the substance in warm Ringer's solution.

- The ionophore, A23187, was prepared by making stock solutions of the substance in methyl alcohol. The stock solution was stored at -5°C in a bottle wrapped with aluminum foil (to protect the contents from light). The drug solution was appropriately diluted with Ringer's solution and used whenever necessary.

All other drug solutions were prepared fresh in Ringer's solution and in all cases the pH was adjusted between 7.2 and 7.4.

3.1.3 Isotope, Tissue Solubilizer and Liquid Scintillation Fluid:

The isotope of Ca^{++} used was ^{45}Ca obtained from New England Nuclear Canada Ltd. as the chloride.

The scintillation fluid used to measure β-emission from ^{45}Ca was ACS, a commercial xylene-surfactant based aqueous counting scintillant from Amersham.

The tissue solubilizer used was NCS, a commercial product consisting of a quaternary ammonium base in toluene, from Amersham.

3.2 METHODS:

3.2.1 Tension Studies:

3.2.1.1 Muscle Preparation:

The extensor longus digiti IV (toe) muscles of the frog *Rana pipiens* were used in all experiments involving tension studies. Each frog was decapitated and pithed and the muscle dissected and removed. After removal, the muscle with each of its tendons tied to a silk thread was placed in a glass petri dish containing the appropriate physiological solution. The silk threads were then wrapped around two plastic pegs which were stuck to the inner surface of the dish close to its circumference and at diametrically opposite ends. This helped in positioning the muscle in the petri dish and facilitated further dissection. Care was taken not to stretch the muscle excessively. With the aid of a dissection microscope (Wild-Heerbrugg, Switzerland) the muscle was freed from connective tissue and fascial membranes. All muscles were allowed to equilibrate for a period of 40-60 min following dissection in the physiologic solution before an experiment was commenced.

3.2.1.2 Muscle Chamber:

. The muscle chambers used for tension recordings were constructed from glass or plastic barrels of syringes of 3 or 5 ml capacities. The toe muscle was mounted vertically in the chamber between a glass hook and a strain guage. The proximal end of the muscle was secured to the glass hook near the bottom of the bath and the distal end was attached to the arm of the strain guage positioned above the bath, by means of the silk threads. The position of the strain guage was adjusted using a micromanipulator. This was done in a manner so that an optimum twitch was obtained and that the slack in the muscle was taken up but no significant load tension (<100 mg) was applied. The physiological solution bathing the muscle was continuously bubbled with a gas mixture of 99.5% O_2 and 0.5% CO_2. The solution in the chamber was changed by draining at the bottom and by introducing fresh solution at the top.

3.2.1.3 Stimulation parameters:

The toe muscles were stimulated by means of two ring electrodes of platinum situated near the top and bottom of the solution in the muscle chamber. For single twitches, pulses of 0.5 msec duration were used once every 30 sec. For summated responses the number of pulses and the pulse intervals were varied between 2

and 10, and 0.5 and 100 msec respectively. For tetanic
stimulation, the stimulus frequency was increased in
steps of 10 Hz from a starting value of 10 Hz until a
fused tetanus was obtained. In most cases a frequency
of 60 Hz was adequate to produce a maximal tetanus.
Generally, a 5-10 sec train of pulses was used at the
appropriate frequency to obtain the maximal tetanic
response. In all cases pulses of 0.5 msec duration and
of supramaximal strength (voltage) were used.

3.2.1.4 Tension Recording:

Tension was recorded by means of pixie transducers
(Endevco Model 8121A) in a wheatstone bridge
configuration. The rate of change of tension during
the twitch was determined in some experiments by
electrical differentiation. This was achieved by
introducing a capacitance (0.003 microfarads) into the
output of the DC amplifier. The output from the
transducer and its first differential (Fig. 3) were
recorded using separate channels of a Gould (Brush)
2200 pressurized ink recorder. Calibration of the
differential signal was achieved by determining the
slope of the tangent drawn through that point on the
tension trace where the rate of change is maximal; this
point being the point of intersection on the tension
recording of a line drawn vertically through the peak
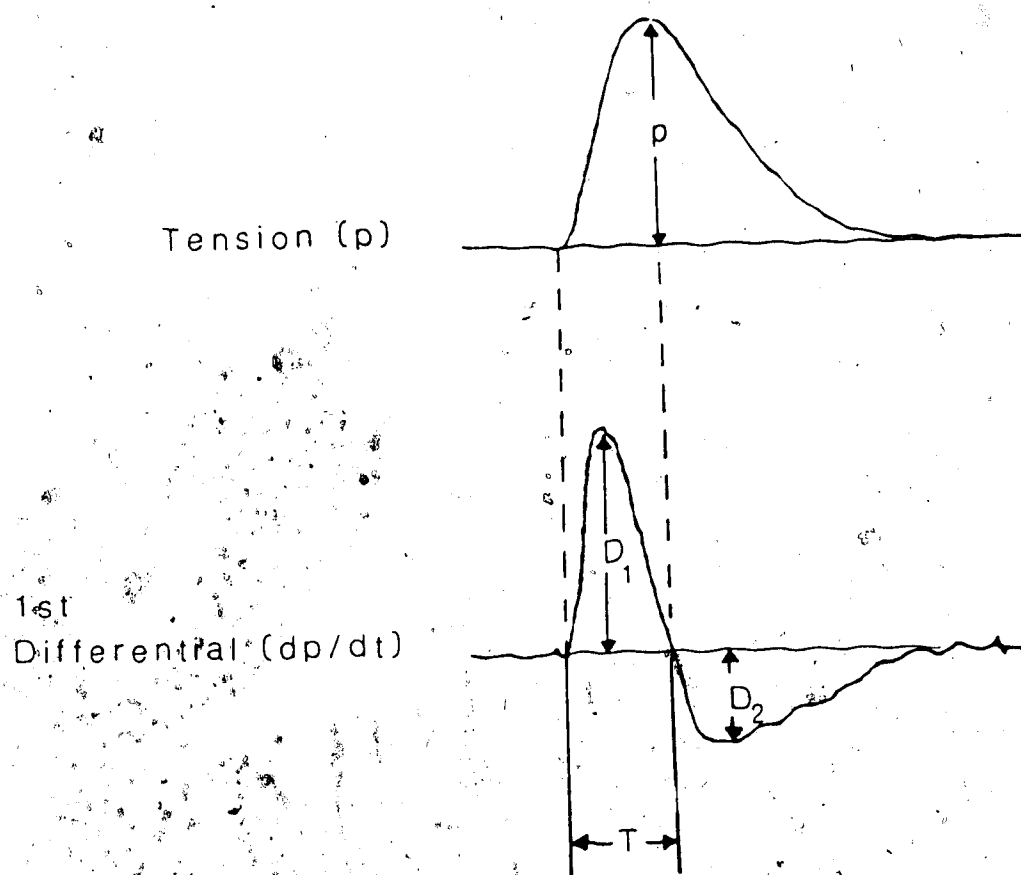
Figure 3. A typical recording of a twitch tension (upper
tracing), and its 1st differential (dp/dt) (lower
tracing). P, peak twitch tension; D_1, maximal value of
dp/dt (i.e., the maximal rate of rise); D_2, minimal
value of dp/dt (i.e., the maximal rate of fall); T,
time to peak tension.

of the differential recording.

3.2.1.5 General Experimental Protocols:

The toe muscles were excised, mounted and stimulated (once every 30 sec) as described above. If the height of the 'control' twitches remained steady for 30 min, the tissue was considered suitable for an experiment.

In some experiments the effects of opioids were studied on the responses to closely spaced multiple electrical stimuli. The toe muscles were subjected to trains of closely spaced electrical pulses; each train of stimulus comprising of 2, 3, 5, 7 or 10 pulses. The inter-pulse time interval (pulse interval) in each of these trains was increased from 0.5 msec to a maximum of 100 msec and from the numerous responses recorded, the optimally summated and completely fused response (maximally summated response) was identified. Generally, under drug-free conditions, a pulse interval of 3-6 msec was effective in producing such a maximally summated response. The responses thus obtained under 'control' and under 'test' conditions were then compared. Often, it was not possible to identify the maximally summated response by examining the tension recordings alone. In such cases, the 1st differential of the tension profile, recorded simultaneously, served

as a sensitive indicator of completely fused responses (Fig. 4). The response having the largest maximal rate of rise and with the largest amplitude was considered as maximally summated. Summated responses obtained with pulse intervals slightly greater than that required for the maximally summated response showed differential traces with a smaller maximum rate of rise (D_1) and were often characterized by notches along their paths. Such responses were therefore considered unfused and none of them was labelled as the maximally summated response even though its amplitude might have been greater than that of the designated maximally summated response.

The criteria described above were satisfactory in aiding in the identification of the maximally summated response to trains of either 2 or 3 pulses but for trains with a larger number of pulses they had to be extended somewhat. With trains of 5, 7 or 10 pulses and with increasing pulse intervals there was a transition of the muscle response from the maximally summated response into an unfused tetanus (Fig. 5). In this transition region it was not always possible to distinguish the former from the latter using the tension recording alone. Again the differential trace was used in addition to the tension trace to identify the maximally summated response under these conditions.

500 mg

40 mg/msec

Single Pulse

Double Pulses

b   c   d   e   f   g

500 msec
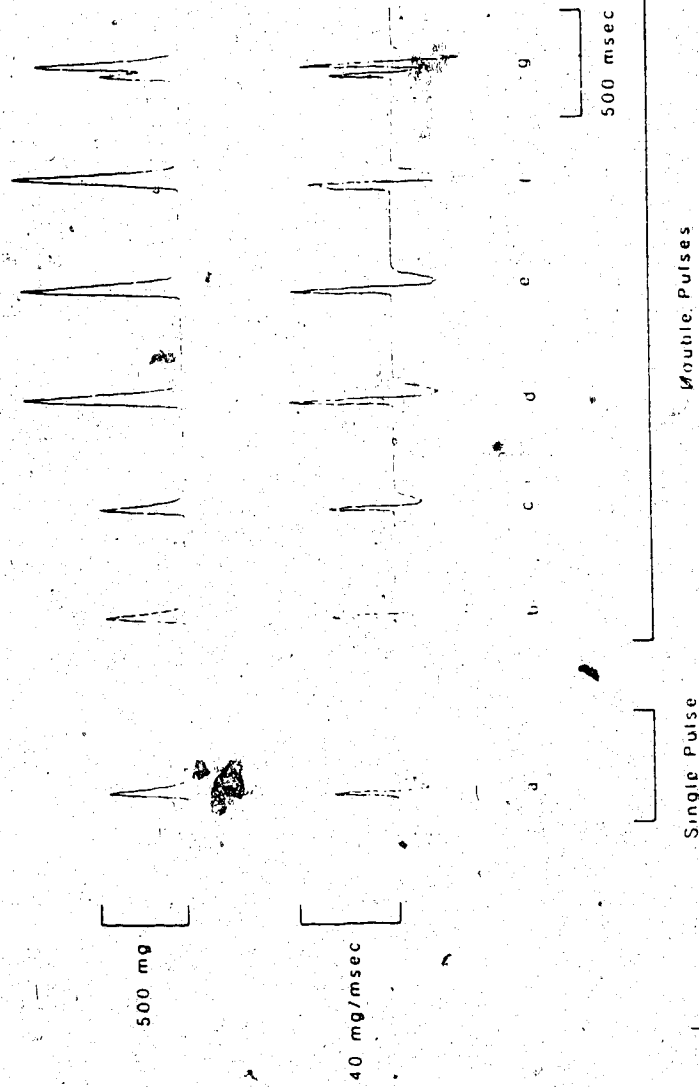
Figure 4. Recordings of twitch tensions and their 1st differentials following electrical stimulation with single or double pulses. Upper recordings represent tension changes and the lower recordings are the corresponding 1st differentials. a, response to a single electrical pulse of duration 0.5 msec and of supramaximal voltage: b - g, responses to closely spaced double electrical pulses each of duration 0.5 msec and of supramaximal voltage. The time interval between the two pulses of the double-pulse train was increased progressively from b to g: b, 1 msec; c, 2 msec; d, 3 msec; e, 4 msec; f, 20 msec; g, 50 msec.
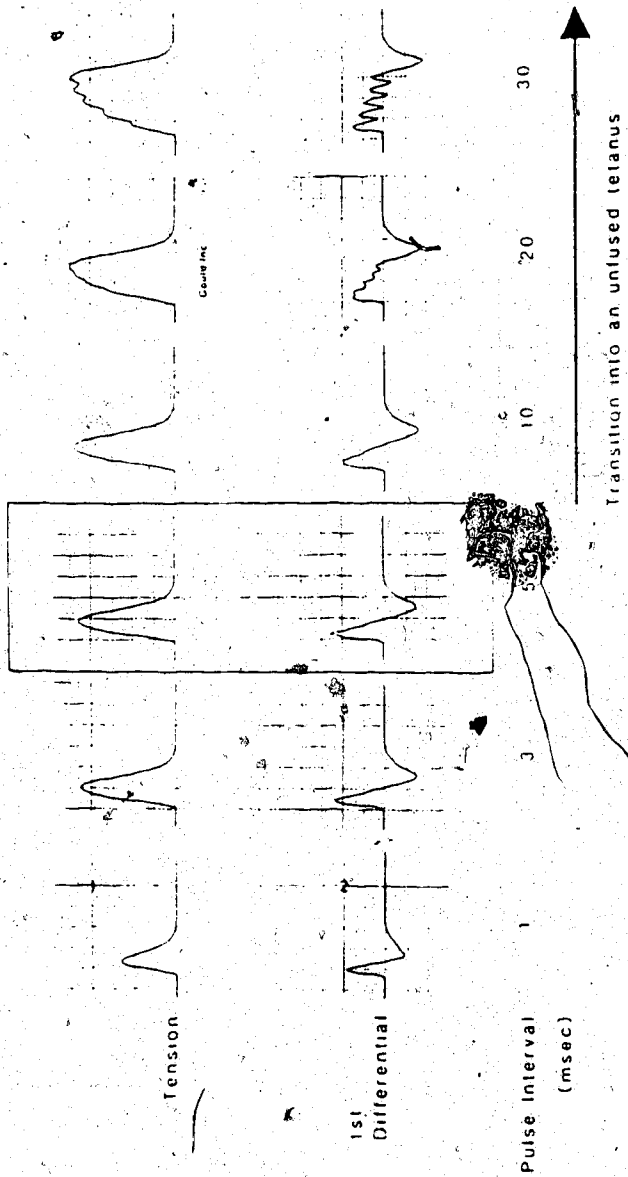
Figure 5. Identification of the maximally summated response of the frog's toe muscle following its stimulation with trains of 5, closely spaced, electrical pulses. The muscle was stimulated with 0.5 msec supramaximal pulses. The pulse interval was increased progressively from 1 msec to 30 msec as indicated.

The response having the differential tracing with the largest rising phase followed closely by the falling phase and also having the highest tension profile was considered to be maximally summated, whereas a differential tracing with a smaller maximum and/or a delayed falling phase indicated transition into an unfused tetanic response (Fig. 5). Generally such differential tracings also showed small plateaus at their peaks and/or notches along their paths reiterating the unfused nature of the responses.

The other characteristics of the maximally summated responses that were examined for opioid effects include the maximum rates of rise/fall of tension and the times to reach peak tension. The values of these parameters were obtained from the associated differential traces. The heights of the +ve (D_1) and the -ve (D_2) peaks of the differential trace from the original resting level were measures of the maximum rates of rise and fall of tension respectively, and the time to peak tension (T), was measured from the start of the response to the time on the differential trace where the rate of change of tension is zero (Fig. 3).

In other experiments the effects of various opioids on muscle contraction were investigated using twitches evoked once every 30 sec by single pulses of

duration 0.5 msec and of supramaximal strength. A concentration-effect curve for each opioid was determined by adding increasing concentrations of the compound and noting the maximum response for each concentration. Routinely, drugs were applied to the organ baths containing the muscle until the response plateaued, at about 10-15 min intervals. The bath was flushed 3 to 4 times in between drug additions with Ringer's solution. Thus in all these experiments cumulative doses were employed and the application of each drug concentration was preceded by a new control.

For drug antagonism studies, two or more 'control' responses of the muscle to the agonist were initially obtained. The Ringer's solution containing the agonist was then removed from the bath by draining and it was replaced with fresh (drug-free) Ringer's solution. The muscle was repeatedly flushed (once every 3-4 min) with fresh Ringer's solution until the drug-effects were reversed. The muscle-responses to electrical stimulation were then recorded in the presence of the antagonist which was allowed to remain in the bath for a specific period of time (10-45 min). Finally, the agonist was added and the twitch-responses of the muscle were recorded in the presence of a combination of the agonist and antagonist.

Finally, for studying opioid-effects on K^+-induced contractures, two or more control responses of the muscle to a high-K^+ Ringer's solution ($[K]_o = 25$ mM) were initially obtained. Each of the exposure periods to the above solution was kept short viz., 30 - 45 sec so as to minimize muscle swelling by any electrolytic imbalance. A minimum time-period of 15 min was allowed between consecutive testings to enable full recovery of the muscle from the high-K^+ effects. The response of the muscle to elevated K^+ was next recorded in the presence of the opioid and it was compared with the 'control' responses obtained earlier,

3.2.1.6 Analysis:

Measurements were taken from the recordings made on chart paper. Results were routinely expressed as a percentage of the 'control' (100%) response. In some cases quantitative data have been presented for an individual representative experiment even though the experiment itself had been performed more than once. This was done with the intention of illustrating the general trend observed in such experiments.

Student's t-test or ANOVA followed by Newman-Keuls multiple range test was used for evaluation of differences between 'control' and 'test' values. The difference between groups was considered significant

when p < 0.05.

For statistical comparison of a percent change in a twitch tension with its control, the t-value was calculated using an expected value (μ) of 100%. One-tailed t-tests [251] were used in those cases wherein we were interested in testing the statistical significance of only the observed twitch potentiation. In those cases where no multiple comparisons were made with a single control, two-tailed t-tests were carried out for each pair of drug combinations. For multiple comparisons with a single control, an analysis of variance (ANOVA) was carried out. If a significant F resulted from the ANOVA, then the Newman - Keuls multiple range test was applied to determine where significant differences existed between the means.

3.2.2 Electrophysiological Studies:

Experiments were performed at room temperature (20°C) with the sartorius muscle isolated from the leopard frog, *Rana pipiens*. The experimental procedures used in this study have been described previously [252, 12, 14].

3.2.2.1 Muscle preparation:

The sartorius muscle along with a portion of the pelvic girdle containing the proximal attachment of the muscle was carefully dissected and removed from a decapitated and pithed frog. The muscle was then placed in a dissecting dish and, with the aid of a dissection microscope, was freed from connective tissue and fascial membranes. The whole muscle was mounted horizontally in a lucite bath containing Ringer's solution in such a way that the deep surface of the muscle lay uppermost. This was important, since the superficial surface is covered with a layer of connective tissue which hinders the insertion of microelectrodes [253]. For final mounting, the muscle was stretched approximately 10% of slack length over two glass posts and then firmly pinned down in the bath. The stretch prevented damage to the microelectrode tips and the myofibre membrane upon movement of the muscle and also minimized movement artifacts. The bath was illuminated from below and

viewed from above with a binocular dissecting microscope (Wild-Heerbrugg, Switzerland).

Some of the frog muscles were found to be infected with larvae of nematodes and trematodes. Muscles found to be heavily infected were discarded, whereas those that were relatively less infected were used after removing the visible parasites and the proliferating connective tissue layer around them [254].

3.2.2.2 Stimulation and recording system:

A small proportion of the muscle fibres were stimulated by an extracellular, bipolar, platinum-filled pore electrode (tip diameter = 0.4 mm) placed on the surface of the muscle 5-10 mm from the recording electrode. In most instances the muscle fibres were stimulated initially by 0.5 ms single pulses of 0.3 - 0.5 V delivered through a stimulus isolator unit (WP Instruments, model 305 B). The fibres being electrically stimulated could be identified by their movement in response to the stimulus. The recording microelectrode was then lowered into one of those fibres for the purpose of recording the various electrical parameters of the myofibre. Conventional glass capillary microelectrodes filled with 3 M KCl and having external tip diameters less than 0.5 μ and resistances between 10 and 40 M ohm

were used as intracellular recording electrodes. A broad-band electrometer (WP instruments, Model M-4) was used to measure membrane potentials. The negative capacity feature of this instrument was useful for the compensation of stray input capacitance. The X1 or X5 output of the electrometer was connected to an oscilloscope (Tektronix, Type 565, or Nicolet 3091). The reference electrode consisted of a chlorided silver wire, formed into a spiral, and placed in the solution bathing the muscle.

The resting membrane potential and the action potential were recorded using two oscilloscope beams. The rate of change of the membrane potential during an action potential was determined by electrical differentiation. This was achieved by introducing a capacitance (20 picofarads) connecting the inputs to the two DC amplifiers on the oscilloscope. This differentiation circuit had a time constant of approximately 20 μsec, and provided an output voltage proportional to the rate of change of input [255]. Calibration was achieved by introducing a saw-tooth wave into the input of the differentiating circuit. Measurements were taken from enlarged photographic records of the oscilloscope traces.

Some of the later experiments were performed using a digital oscilloscope (Nicolet 3091). In these cases,

the experimental procedure was the same except for the computerised processing of the data. The layout of the assembly used for the intracellular measurements is shown in Fig. 6. The traces of the action potential and its first differential (Fig. 7) were stored in the memory of the digital oscilloscope and then were transmitted immediately to a micro-computer (Kaypro II) which determined and printed the values of the parameters of interest, viz., the resting membrane potential, the action potential amplitude, duration of the action potential at any pre-defined level of membrane potential, and the maximal rate of rise and fall of the action potential.

With either recording oscilloscope the data were typed into another microcomputer (Hewlett Packard 9816) which processed it by performing statistical evaluations and by plotting the results on paper using a plotter (Hewlett Packard 7470 A). Traces from the digital oscilloscope after D to A conversion were periodically copied onto paper using an X-Y chart recorder (Hewlett Packard 7015 B).

3.2.2.3 Recording electrodes:

Recording electrodes were made from open-ended glass capillaries (Fisher Scientific Co.) having 1.5 - 2 mm outside diameter. The microelectrodes were drawn
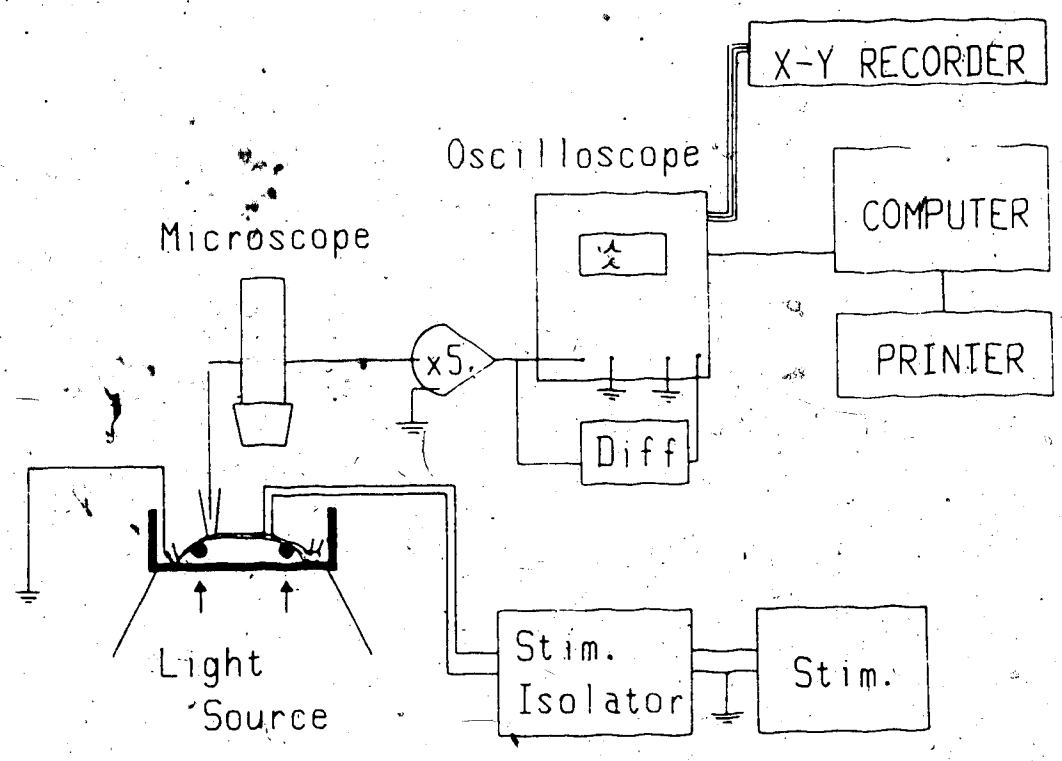
Figure 6. Diagrammatic illustration of the assembly used for intracellular measurements.
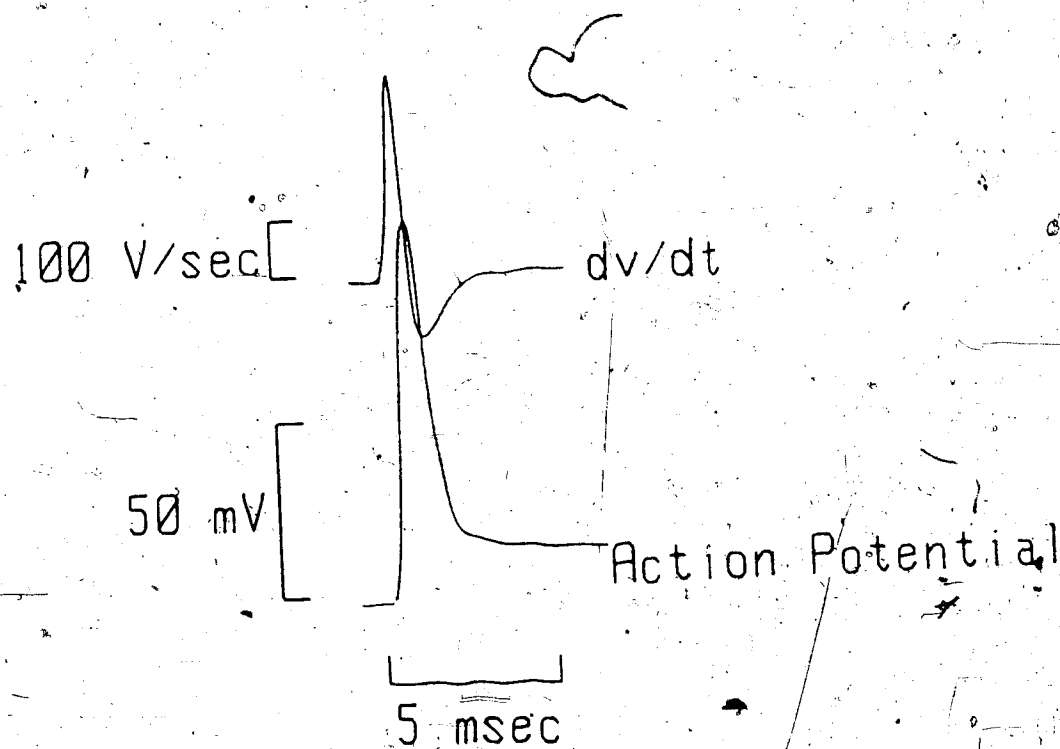
Figure 7. Typical recordings of the action potential and its first derivative (dv/dt) from the frog's sartorius muscle.

by a glass microelectrode puller (PN-3 Narishige Scientific Instrument Lab. Ltd., Japan). These microelectrodes were then mounted on microscope slides with a rubber band wrapped around them and placed in a coplin staining dish with their tips downward. The coplin dish was then filled with methanol and placed in a vacuum dessicator. The methanol was allowed to boil for about 5 min by reducing the pressure in the dessicator. With this procedure, almost all the electrodes were completely filled with methanol. The slides with the electrodes were then transferred successively into two coplin dishes each containing distilled water and they were left in each dish for 4 - 5 hrs. Finally, the electrodes were placed in 3 M KCl solution and left there overnight before use. Only microelectrodes having resistances of about 10-40 M ohm were used. A solid state volt-ohm-microamp meter (Danameter, Dana Lab. Inc., Irvine, California) was used to measure the resistance of the microelectrodes.

3.2.2.4 General Experimental Protocols:

Electrical events were recorded from (usually) 6-8 surface fibres over short time periods (about 2 min overall). Control recordings were taken after an equilibration period of 60 min. The tissue bathing medium (Ringer's solution, pH 7.2 - 7.4) was replaced

with fresh solution once every 15 min during the equilibration period. After the 'control' readings were obtained, the tissues were exposed to the drug dissolved in Ringer's solution and traces of the electrical events were obtained at frequent time intervals, viz., 3 - 5, 8 - 10, 18 - 20 and 28 - 30 min following exposure to the drug and the readings obtained constituted respectively the results for the .5, 10, 20 and 30 min 'tests' described in the Results section. Since these intracellular recording experiments were performed to study changes that might explain the twitch-potentiating effect of opioids which has a rapid onset of action (< 30 sec) and which develops fully within 5-10 min of drug exposure, the drug-effects on the resting and action potentials were studied for only 30 min after drug addition.

The opioids tested for their effects on the electrical properties of the frog skeletal muscle fibre were dextrorphan ($10^{-5} - 3 \times 10^{-4}$M), etorphine ($10^{-5} - 10^{-3}$M), naloxone ($10^{-4} - 10^{-3}$M) and methadone ($3.2 \times 10^{-4}$M). The opioids were tested in concentrations that produced discernable twitch potentiations in the frog toe muscle. Etorphine, a potent opioid lacking the twitch-potentiating ability was also tested in order to delineate common opioid effects on the electrical properties of the muscle fibre membrane unrelated to

the twitch potentiating effect.

For drug antagonism studies the electrical responses of the muscle strips were recorded at the start of the experiment and following drug exposure as described earlier. The drug was then removed from the chamber and the muscle was repeatedly washed with fresh Ringer's solution once every 10-15 min until the muscle recovered from the drug-effects as evidenced by the reversal of the drug-effects on the electrical parameters of the muscle. Next, the antagonist dissolved in Ringer's solution was left in contact with the muscle for a fixed period of time (up to 40 min) at the end of which the electrical responses of the muscle were once again recorded. Finally, the tissue was left in contact with a combination of the agonist and the antagonist in Ringer's solution, and the electrical events were recorded for the next 30 min as described earlier. Methadone (3.2×10^{-4} M) was the only opioid agonist whose effects were tested for antagonism by high-Ca^{++} Ringer's solution ($[Ca]_0 = 8.64$ or 10.08 mM).

3.2.2.5 Analysis:

The effect of any given concentration of an opioid on action potential production was evaluated by determining the mean values of the maximum rates of rise (dv/dt) (% of predrug control) as a function of

time. To evaluate the effect of an opioid on rectification of the action potential, the maximum rate of fall was similarly determined as a function of time. Since the maximum rate of rise of the action potential is proportional to the positive current entering the muscle fiber during the rising phase of the action potential, this parameter estimates the function of the Na^+ conductance processes. Similarly, since the maximum rate of fall of the action potential is proportional to the positive current leaving the muscle fiber during the falling phase of the action potential this parameter can be used to estimate the function of the potassium conductance processes [255, 230].

To evaluate opioid-effects on the current flowing through the cell during an action potential, the duration of the action potential (measured at -40mV, an arbitrarily chosen value) was determined as a function of time. Prolongation of the duration of the action potential has been suggested to produce an intensification and prolongation of the active state of the muscle [256].

Student's t-test or ANOVA followed by Newman-Keuls multiple range test was used for evaluation of differences between the 'control' and 'test' values. The difference between groups was considered significant when $p < 0.05$. For multiple comparisons

with a single control, an ANOVA (analysis of variance) was carried out. If a significant F resulted from the ANOVA, then the Newman-Keul's multiple range test was applied to determine where significant differences existed between the means.

3.2.3 Radioisotopic Studies:

3.2.3.1 ^{45}Ca Desaturation Studies:

^{45}Ca desaturation studies were conducted to investigate the effects of opioids on the surface membrane bound Ca^{++} of frog's skeletal muscle fibres.

Muscle Preparation:

All experiments in this study were performed with extensor longus digiti IV (toe) muscles isolated at room temperature from frogs (*Rana pipiens*). The muscles were dissected very carefully and placed in petri dishes containing the appropriate physiologic buffer (bicarbonate or Tris-buffered Ringer's solution) with or without $CaCl_2$. All muscles were allowed to equilibrate for at least 1 hr following dissection in a non-radioactive physiological solution before an experiment was commenced.

In all experiments both toe muscles; one from each foot of an individual frog, were used with one toe muscle serving as the control. These constituted the muscle pairs referred to in the Section on 'Results' (Ch. 4.3.3.8).

Experimental Set Up:

Briefly, the experimental approach involved the use of ``Ca which was first allowed to accumulate in the preparation during the accumulation period (0.5 - 5 hr).   The   preparation was next transferred to a tracer-free medium which would result in a net efflux of the tracer. This medium was assayed for the radioactivity released from the muscles at different times by transferring the preparation successively to different vessels containing the medium every 1 or 5 min and then measuring the radioactivity in each vessel.

The toe muscle of the frog contains about 20-50 muscle fibres and in the resting state has a length of about 1.5 - 2.0 cm and a maximum diameter of 0.5 - 1.0 mm. Since this muscle is very light (weight <1.5 mg) it tends to float on the surface of any physiologic buffer on which it might be placed.   To ensure optimal efflux from the tissue it is necessary to have it totally immersed in the efflux medium.   To facilitate rapid transfer of the tissue from one efflux beaker to another and to ensure prompt and complete immersion of the light toe muscle in the efflux solution a modification in the standard efflux technique [257, 258] was devised. The modified experimental setup consisted of the

following components: tissue hook, tissue transporter, efflux containers and an electromagnetic device (Fig. 8).

The tissue hooks were made from minute insect pins having a diameter of 0.15 mm and a length of 10 mm. The sharp end of the pin was bent to form a 'V' shaped hook and the other end was rolled to form a ring. New tissue hooks were used for each experiment and were disposed off at the end of the experiment. The tissue transporter consisted of a rectangular block of perspex (4 x 3 x 0.25 cm) provided with a handle. On either side of the handle and at a distance of 1 cm from it were two holes (0.1 cm O.D.) through each of which passed a steel rod. The lower portion of each rod was bent to form a 'U' shaped hook. The vertical position of each of the rods could be adjusted manually using set screws. The efflux was carried out in disposable polystyrene microbeakers of 5 ml capacity. Rows of beakers were placed in a horizontal rack with sufficient clearance at the bottom to accommodate an electromagnet between the beaker and the work-table. The electromagnetic device comprised of an electromagnet (6 V) connected to an appropriate power supply. The electromagnet could be positioned beneath any one of the efflux

Electromagnet
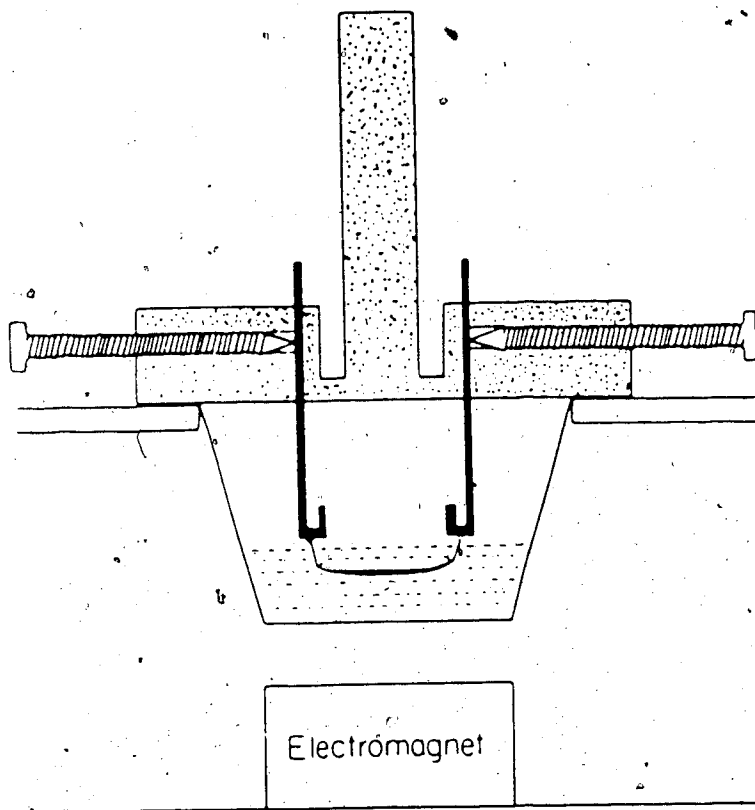
Figure 8. A diagrammatic sketch of the apparatus used in
⁴⁵Ca efflux studies. The plastic tissue transporter
(dotted fill) had a handle at the top, and two set
screws for adjusting the position of the two large
hooks. The fine steel tissue hooks attached to each
end of a toe muscle were placed on these larger hooks
as shown. Also illustrated is an efflux beaker with
the toe muscle submerged in the efflux fluid and the
position of the electromagnet below the efflux beaker.

beakers and could be activated by touching a switch on the power supply.

To effect ⁴⁵Ca efflux, the tissue transporter (carrying the radioactively labelled muscle) was placed on the rack over the collar of the efflux beaker as shown in Fig. 8. Care was taken to see that the large steel hooks of the tissue transporter did not touch the efflux solution. To overcome the tendency of the toe muscle to float on the surface of the efflux solution in the beaker, it was necessary to draw the tissue hooks towards the bottom of the efflux beaker thereby ensuring the complete immersion of the tissue in the efflux fluid. This was achieved by activating the electromagnet just prior to the movement of the tissue into the efflux beaker thereby aligning the minute tissue hooks in the downward direction. This resulted in a smooth immersion of the toe muscle in the efflux medium. The magnet was then immediately inactivated and positioned beneath the next efflux beaker.

General Experimental Protocols:

Following the incubation in the nonradioactive physiological solution, the muscles were incubated for a fixed period of time (0.5 - 5 hr) with the

same solution containing 4 - 5 $\mu Ci/ml$ of ^{45}Ca. Throughout the incubation period the muscles were bubbled with a gas mixture of 99.5% O_2 and 0.5% CO_2. The tendons were then carefully cut from the muscle and through the connective tissue at either end of the muscle fibre bundle the sharp end of the tissue hook was inserted. The tissues were then rinsed by two rapid dippings (2 sec each) in 5 ml of the nonradioactive and Ca^{++}-free physiological solution to remove any loosely adhering ^{45}Ca. Ca^{++} was omitted from the rinse solution to prevent removal of any self-exchangeable Ca^{++}. The tissue hooks with the tissues were then suspended from the tissue transporter and passed through a series of beakers containing 2.5 ml of the efflux medium, viz., Ca^{++}-free physiologic solution with or without the drugs being tested. The muscles remained in each beaker for an accurately measured period of time (1 min or 5 min).

At the end of the final efflux period the tissues were blotted for about 10 sec by gently rolling them over medium-fine porosity filter papers (Whatman No. 2) that had been previously moistened with a few drops of the efflux medium. The tissues were then transferred into disposable plastic scintillation vials and solubilized. The

solubilized muscles as well as 2 ml aliquots of the efflux medium were next assayed separately for radioactivity. The counts/min observed in each sample of the efflux medium was corrected for background radiation and quenching.

The experimental procedure was varied with respect to parameters such as the composition of the incubation/efflux medium' or the duration of the incubation/efflux. The modifications in individual sets of experiments are described in the Chapter on 'Results' (Ch.4.3.3.8).

Tissue Solubilization:

At the end of the efflux period the tissues were blotted and transferred into plastic scintillation vials containing 0.5 ml of a tissue solubilizer (NCS, Amersham). To this was added 0.1 ml of distilled water and the mixture was digested overnight at 50°C in a shaking water bath. The contents of the vial were then cooled to room temperature and 17 μl of glacial acetic acid was added to neutralize the mixture and prevent chemiluminescence.

Radioactivity Counting and Quench Correction:

2 ml of the radioactive sample from the efflux beaker was pipetted into a plastic scintillation

vial and to it was added 15 ml of the scintillation cocktail (ACS, Amersham). The mixture was shaken and dark-adapted overnight. The solubilized tissue too was mixed with 15 ml of the scintillation cocktail and dark-adapted as above. The radioactive sample was then counted for radioactivity in a Beckman Liquid Scintillation Counter (LS 6800). A set of 'blanks' was run, both, for the efflux samples and the solubilized tissue samples, and the radioactivity in the respective samples was corrected for background radiation.

Quench was monitored by H-number. Samples were counted with H-numbers and were subjected to quench correction in order to determine actual sample activity - disintegrations per minute (dpm) - regardless of quenching in the samples. Quenched standards containing a known activity of ^{45}Ca and varying amounts of carbon tetrachloride, the quenching material, in 2 ml of Ringer's solution were prepared and counted to determine count rates - counts per minute (cpm). Similar quenched standards but in 0.5 ml of the solubilizer (instead of 2 ml of Ringer's solution) were also prepared and counted as above. H-numbers were measured for each standard and a quench calibration curve relating counting efficiency of each standard to its corresponding

H-number was generated.

Counting efficiency is the ratio between observed cpm (corrected for background radioactivity) and the actual dpm:

Counting efficiency = cpm / dpm

Knowing the counting efficiency, the disintegration rate is given by:

dpm = cpm / counting efficiency

The counting efficiency of the unknown was obtained by interpolation of the quench curve formed with the standards. This interpolated efficiency value was used in the above equation to obtain sample dpm.

Calculation and Presentation of Data:

Successive addition of the disintegrations per min (dpm) in the samples to the dpm remaining in the preparation at the end of the experiment provided the radioactivity present in the preparation at different sampling times. Data were expressed as either the percentage of ^{45}Ca remaining in the muscle after each washout interval (desaturation curve) or as rate coefficient plots which express the decline of tissue ^{45}Ca content as a percentage of that ^{45}Ca present in the tissue during each washout time interval (percentage of ^{45}Ca lost per minute) [258, 259]. Student's t-test was used for

evaluation of differences betwen 'control' and 'test' values. The difference between groups was considered significant when P <0.05.

3.2.3.2 Ca^{++} Uptake Studies:

Preliminary experiments were carried out to determine the effects of methadone on Ca^{++} uptake during the twitch. The experimental procedure used to determine Ca^{++} influx was essentially similar to that employed by Bianchi and Shanes [260], except for the adaptation of the lanthanum wash procedure [261]. The latter modification was necessary because changes in intracellular Ca^{++} pools could only be detected after displacement of the large membrane bound Ca^{++} compartment with La^{+++}.

Muscle Preparation:

All experiments in this study were performed with extensor longus digiti IV (toe) muscles isolated from frogs (Rana pipiens) at room temperature (20°C). The muscles were dissected very carefully and placed in petri dishes containing Tris-buffered Ringer's solution. All muscles were allowed to equilibrate for at least 1 hr following dissection in the physiological solution before an experiment was commenced. In all experiments, both toe muscles, one from each foot of an individual

frog, were used with one toe muscle serving as the control. These constituted the muscle pairs referred to in this and subsequent chapters.

Experimental Setup:

The experimental setup for eliciting and recording twitches was the same as that used earlier for tension studies (Ch.3.2.1.2., and 3.2.1.4), excepting for the additional use of a close fitting lid at the mouth of the muscle chamber. This helped prevent spraying of the radioactive incubation solution into the surrounding regions during bubbling of the solution with the gas mixture of 99.5% O_2 and 0.5% CO_2. The lid, however, had a slit to accommodate the tissue holder and allow passage of the silk thread linking the distal end of the muscle to the arm of the strain guage.

General Experimental Protocol:

Following the incubation in the nonradioactive Tris Ringer's solution, the muscles were mounted in a muscle chamber containing the same solution and were electrically stimulated once every 30 sec with pulses of 0.5 msec duration and of supramaximal voltage. If the heights of the twitches of both muscles remained steady for 30 min, they were considered suitable for an experiment.

Briefly, the experimental procedure involved incubation of the muscle in 'hot' Tris Ringer's solution for 10 min under 'control' or 'test' conditions, stripping off the superficially bound Ca^{++} using La^{+++}, and estimating the radioactivity present in the tissue. The radioactive Tris Ringer's solution contained 10 - 15 μCi of ^{45}Ca per ml of the solution.

Two types of Ca^{++} uptake experiments were carried out. The first set of experiments was carried out to see if our experimental procedure is able to detect the stimulation-induced increase in Ca^{++} uptake observed by other investigators [260]. Thus, in four experiments, the net uptake of Ca^{++} per twitch was determined. For this purpose, one muscle of a muscle-pair served as the unstimulated 'control' and the other served as the 'test' muscle. The exposure to 'hot' Tris Ringer's solution was kept short, viz., 10 min because it ensures very little passive uptake and negligible backflux of Ca^{++} [260]. During the second half of this interval the experimental condition of supramaximal stimulation was introduced for the 'test' muscle, while the 'control' muscle was unstimulated. Stimulation was at the rate of one shock per second for a period of 5 min. Therefore a total of 300

shocks were delivered to the 'test' muscle. At the end of the incubation/stimulation period, the radioactive solution in the bath was replaced by Tris Ringer's solution containing 2 mM La^{+++}. The muscle was washed thrice within the first 5 min with the above solution and then once every 5 min for the next 55 min. After 60 min of La^{+++}-wash the muscles were cut free from their tendons, blotted and weighed by difference in an airtight microbeaker using an ultramicrobalance (Mettler, UM3). The weighed tissues were then carefully transferred into scintillation vials for further processing.

In the second set of experiments, the effect of methadone (10^{-4} M) on the stimulated uptake of Ca^{++} was examined. For this purpose, a pair of toe muscles from the same animal were incubated for 5 min in 'hot' Tris Ringer's solution without stimulation. Five sec prior to the end of the above period, 10 μl of a concentrated stock solution of methadone in 'cold' Tris Ringer's solution was added to the 'test' muscle chamber. The concentration of the stock solution was such that it made a final methadone concentration of 10^{-4} M in the bath. An equivalent amount (10 μl) of 'cold' Tris Ringer's solution (drug-free) was similarly added to the 'control' muscle. At the end of the above 5 min

incubation period recurrent electrical stimulation was applied to the muscle. Both the muscles were stimulated at the rate of 1 shock per second for 5 min, as before. The further treatment of the two muscles was as done before (Ch. 3.2.3.1); viz., periodic washings with La^{+++} (2 mM), blotting, weighing and transferring into scintillation vials.

Tissue Solubilization:

The muscles in the scintillation vials were solubilized using a tissue solubilizer (NCS, Amersham) as described earlier (Ch. 3.2.3.1).

Radioactivity Counting and Quench Correction:

The ^{45}Ca activities of the solubilized muscles were determined by liquid scintillation spectrometry, and then corrected for background radiation and quenching as described earlier (Ch. 3.2.3.1).

Calculation:

The radioactivity in 10 μl samples of the incubation medium was determined and the specific activity of ^{45}Ca labelled Tris Ringer's solution was calculated as follows:

Specific activity(dpm/mM) = dpm/ml x 1000/ 1.08

The Ca^{++} uptake was determined as follows:

$$\text{Total Ca}^{++}\text{ uptake} = \frac{\text{dpm (in muscle) x }10^9\text{ x }\dfrac{1}{\text{weight of muscle (mg)}}}{\text{specific activity of incubation solution (dpm/mM)}}$$
(μM/Kg)

The difference between the Ca^{++} retention values in the 'test' and 'control' muscles gave the net uptake following stimulation which when divided by the number of twitches gave the uptake of Ca^{++} per twitch in μM per Kg wet weight.

The difference between the Ca^{++} retention values in the 'control' and 'drug-treated' muscle gave the effect of methadone on the stimulated uptake of Ca^{++} assuming negligible effects of the opioid on passive uptake mechanisms.

Student's t-test was used for evaluation of differences between 'control' and 'test' values. The difference between groups was considered significant when $P < 0.05$.

4. RESULTS

4.1 EXAMINATION OF PHENOMENON:

4.1.1 Single Twitches:

It was earlier reported from our laboratory that meperidine, an opioid agonist, potentiates twitches in the isolated frog sartorius muscle upon direct electrical stimulation [249]. The experiments described in this section were carried out to extend this information further by studying the effects of a variety of opioids on the twitch response of the frog toe muscle. All muscles were stimulated once every 30 sec as described earlier (Ch. 3.2.1.3).

4.1.1.1 Amplitude:

Morphine and most other opioid agonists and antagonists produced a concentration-dependent increase in the twitch height of the directly stimulated toe muscle. Fig. 9 demonstrates the effects of increasing concentrations (10^{-5}, 5×10^{-5}, 10^{-4} or 10^{-3} M) of morphine on the twitch amplitude. The onset of the increase was rapid (< 30 sec), reaching a maximum within 3 to 5 min following the start of the drug exposure. The maximum twitch potentiation produced by the application of the drug was sustained at the lower concentrations (<10^{-4} M) but at higher concentrations (e.g., 10^{-3}) there was a subsequent decrease in the
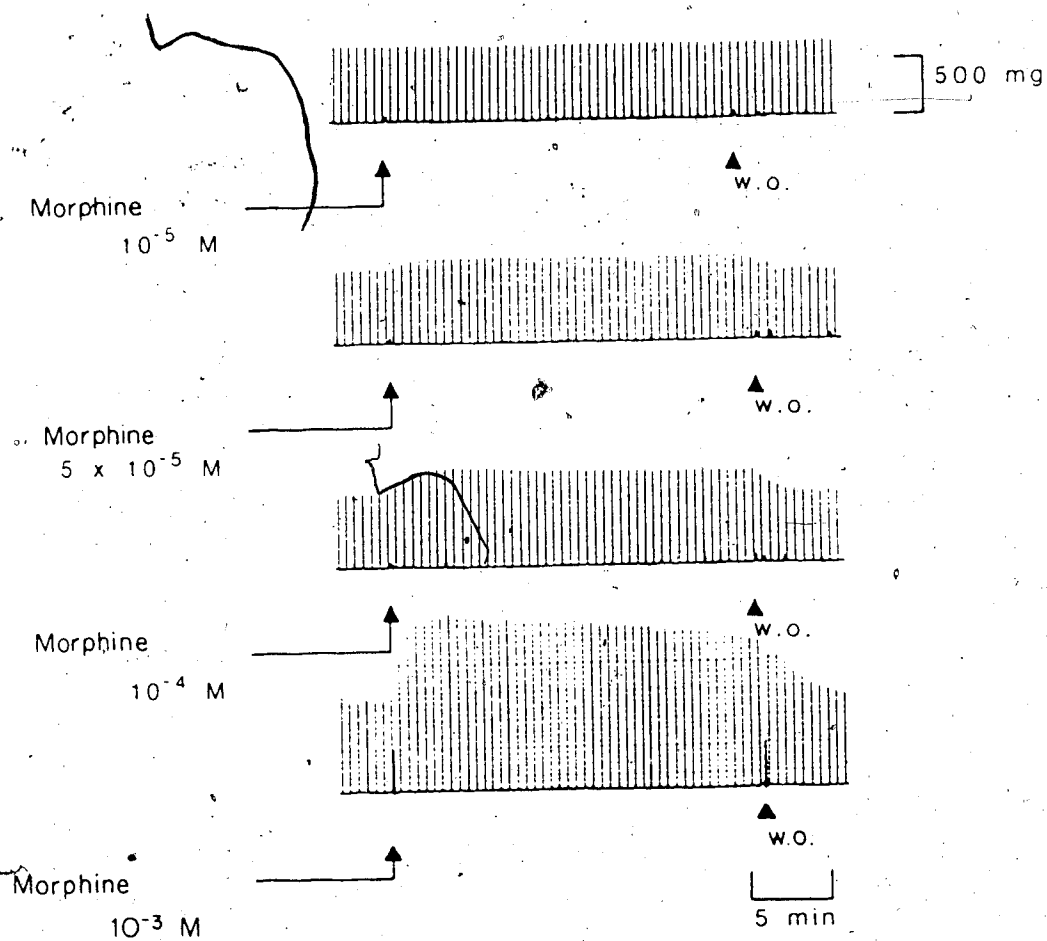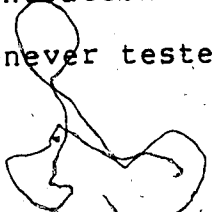
Figure 9. Twitch potentiation produced by morphine in the isolated frog's toe muscle. The preparation was directly stimulated supramaximally once every 30 sec.

twitch height in the continued presence of the morphine. In addition, the twitch-potentiation developed at submaximal concentrations of morphine (e.g., 10^{-4} M) did not attain the magnitude achieved by higher concentrations of the compound even following prolonged contact (>20 min) with the drug. Thus, the twitch potentiating effect of submaximal concentrations of morphine quickly reached its maximum and stayed at that level or decreased slightly with time but never increased in magnitude to match the maximal twitch potentiation produced by a higher concentration of the drug. The reversal of the twitch potentiating effect of the opioid also was rapid upon removal of the opioid agonist.

The other opioids tested produced essentially similar qualitative changes. They caused a potentiating action at low drug concentrations and a potentiation followed by an inhibitory action at high concentrations. Fig. 10 shows the concentration-response relationships for methadone, met-enkephalin and leu-enkephalin. It can be seen that with an increase in concentration above an optimal limit for each compound, the magnitude of the twitch potentiation decreased. This decrease with concentrations above an optimal concentration which produced the maximal effect was found whenever tested; i.e., the response produced
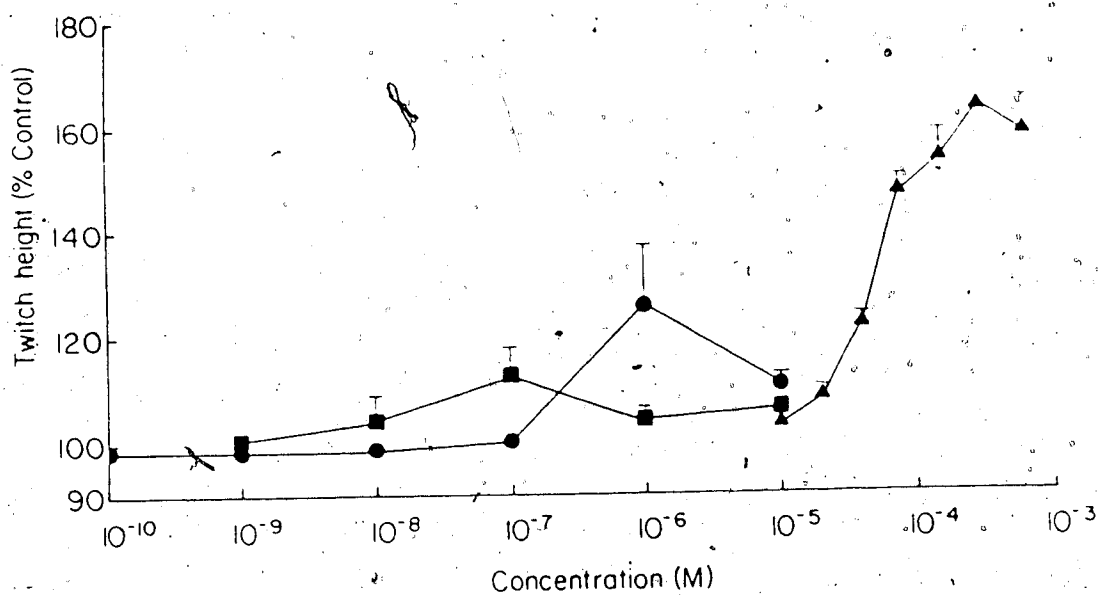
Figure 10. Percent twitch potentiation in isolated frog's toe muscle produced by met-enkephalin (●), leu-enkephalin (■), and methadone (▲) at various concentrations. Each point is the mean ± S.E.M. from six muscles. When the S.E.M. is not shown, it fell within the symbol.

by the highest tested drug concentration was always the maximal response (E max) or less than E max.

The enkephalins generally produced their maximal twitch potentiating effects at concentrations of 100- to 1,000-fold lower than did methadone. However, the twitch potentiations produced by the enkephalins were not consistent and a great deal of variability was observed; e.g., the % twitch potentiation produced by 10^{-6} M met-enkephalin ranged from 100 to 190.9 in different muscles. Also, the enkephalins obtained from Sigma Chemical Company produced very little effect even at high concentrations (up to 10^{-3} M) whereas those obtained earlier from Peninsula Laboratories Inc. produced the twitch potentiations shown in Fig. 10. All the results with enkephalins described in this section were therefore obtained with the enkephalins purchased from Peninsula Laboratories Inc.

The twitch potentiations observed with various concentrations of several other opioids (viz., dextrorphan, levorphanol, (±)morphine, (+)morphine, dextromethorphan, (-)ketazocine, (-)ethyl ketazocine, fentanyl, codeine, naloxone, naltrexone or Mr2096) are shown in Fig. 11 and in Tables 2 and 3. For those compounds available to us in sufficient quantities the concentrations required to produce their maximal twitch potentiations were determined (Table 3), whereas for
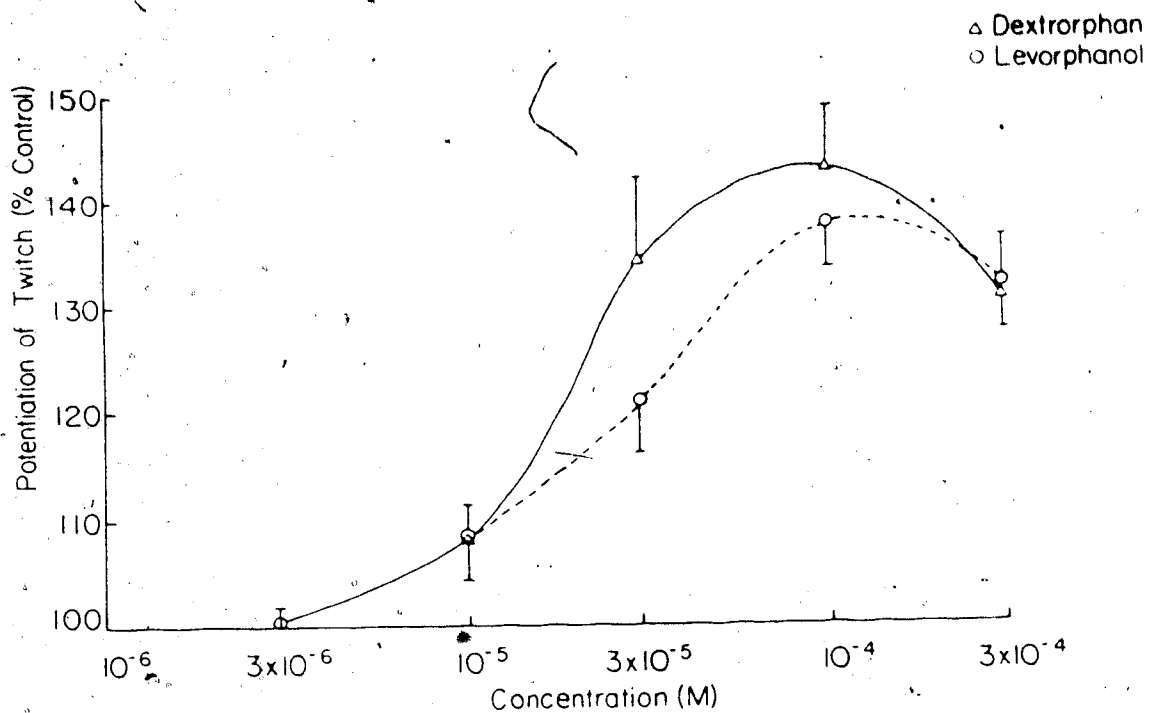
Figure 11. Percent twitch potentiation in the isolated frog's toe muscle produced by dextrorphan (Δ), and levorphanol (O) at various concentrations. Each point is the mean ± S.E.M. from six muscles.

TABLE 2.

Twitch potentiation caused by some opioid drugs at different concentrations in the isolated toe muscle of frog.

Drug	(n)	% Twitch Potentiation at Various Drug Concentrations					
		10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
Morphine (±)	6	100.3 ± 1.3	102.3 ± 1.2	109.0 ± 1.1*	138.9 ± 6.4*	184.3 ± 9.6*	—
Morphine (+)	2	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	125.6 ± 1.1*	—
Dextromethorphan	4	100.0 ± 0.0	100.0 ± 0.0	108.1 ± 4.9	180.4 ± 7.1*	—	—
Ketazocine (-)	6		97.9 ± 2.2	99.3 ± 0.7	134.2 ± 9.7	—	—
Ethyl Ketazocine (-)	6	100.0 ± 0.0	100.4 ± 1.5	100.0 ± 2.4	111.3 ± 5.1*	121.7 ± 4.6*	—
Fentanyl	6	99.2 ± 0.5	99.2 ± 0.5	101.9 ± 0.9	124.9 ± 4.3*	128.8 ± 5.0*	128.3 ± 11.4*
Codeine	6	98.1 ± 1.2	99.7 ± 0.3	100.0 ± 0.0	103.4 ± 1.3	236.7 ± 13.6*	—
Naloxone	6	100.0 ± 0.0	95.7 ± 5.3	94.1 ± 1.8	109.4 ± 1.1*	149.4 ± 3.2*	—
Naltrexone	6	100.0 ± 0.0	100.0 ± 0.0	96.2 ± 3.9	105.0 ± 5.0	—	—
Mr 2096	6	101.2 ± 1.3	—	99.1 ± 1.6	118.6 ± 5.5*	—	—

Note : The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M.
* Significant increases from paired control values adicated: $p < 0.05$. One-tailed, paired sample t-test was applied.

TABLE 3.

The maximal twitch potentiation produced by various opioids in the isolated toe muscle of frog.

Opioid	Tested Concentration Range (M)	Maximal Twitch Potentiation		
		Mean ± S.E.M. (% control)	Concentration Required (M)	(n)
Methadone	1.0×10^{-8} — 6.4×10^{-4}	$163.1 \pm 9.3^{*}$	3.2×10^{-4}	12
Fentanyl	1.0×10^{-8} — 1.0×10^{-3}	$124.9 \pm 4.3^{*}$	1.0×10^{-3}	8
Codeine	1.0×10^{-8} — 1.0×10^{-3}	$128.8 \pm 5.0^{*}$	1.0×10^{-3}	8
Dextrorphan	3.0×10^{-8} — 3.0×10^{-4}	$142.9 \pm 5.5^{*}$	1.0×10^{-4}	6
Levorphanol	3.0×10^{-8} — 3.0×10^{-4}	$137.5 \pm 3.9^{*}$	1.0×10^{-4}	6
Leu-Enkephalin	1.0×10^{-10} — 1.0×10^{-5}	$113.1 \pm 4.9^{*}$	1.0×10^{-7}	6
Met-Enkephalin	1.0×10^{-10} — 1.0×10^{-5}	$126.0 \pm 10.9^{*}$	1.0×10^{-8}	6
Etorphine	1.0×10^{-12} — 1.0×10^{-3}	**	—	8

Note : The values are expressed as a percentage of the control (100%) response.

* Significant increases from paired control responses are as indicated: $p < 0.05$. One-tailed, paired sample t-test was applied.

** This compound produced only a twitch depression at concentrations > 10^{-4} M.

the others the twitch potentiations were obtained at only a few graded concentrations without employing the higher concentrations that would have been necessary to determine their maximal effects (Table 2). The opioid effects on the twitch were found to be quite variable with respect to the degree of potentiation obtained at a given concentration of the drug from muscles of different batches of frogs.

Surprisingly, etorphine (10^{-12} – 10^{-3} M), an extremely lipid soluble and potent opioid-receptor agonist failed to potentiate the twitch in seven out of eight preparations, and in fact caused a depression of the twitch at higher concentrations (>10^{-4} M) (Fig. 12). Also, quaternary naloxone, a compound structurally identical with naloxone excepting for an additional methyl bromide moiety at its tertiary nitrogen atom, produced no twitch potentiation when tested in concentrations up to 10^{-2} M, whereas naloxone, when tested on the same muscles and under identical experimental conditions, produced a marked twitch potentiating effect (Fig. 13).

Experiments were conducted to determine if the electrical stimulus *per se* had any influence either on the time required for the full development of the twitch potentiating effect or on the magnitude of the fully potentiated twitch following exposure to a given
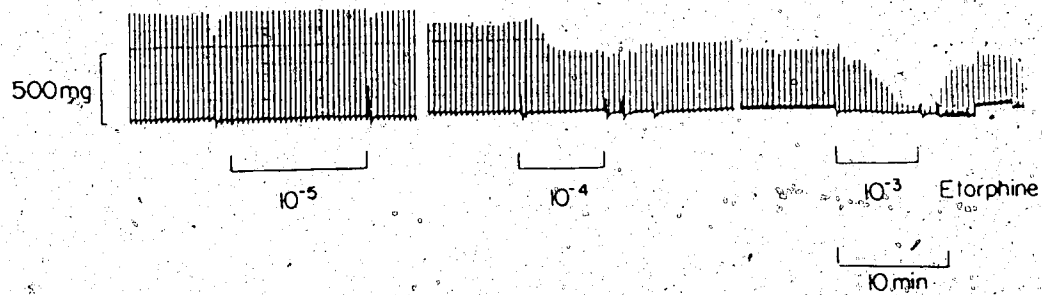
Figure 12. Depression of twitch height by etorphine in an isolated frog''s toe muscle. Horizontal lines below each record show the duration of exposure to the specified concentration of etorphine.
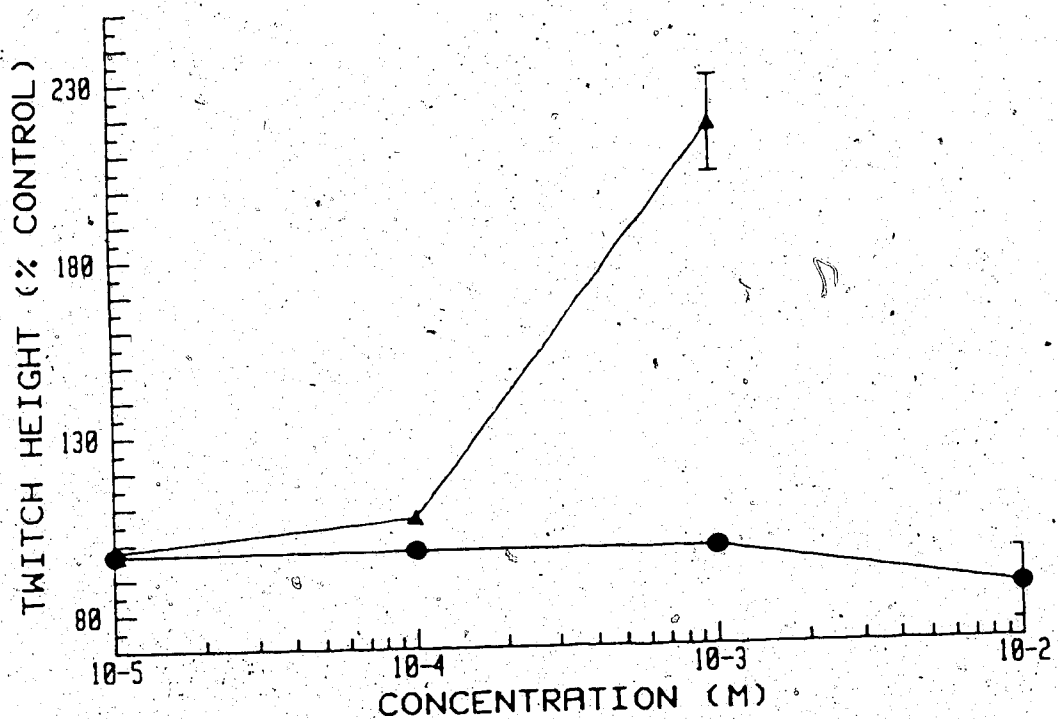
Figure 13. Percent twitch potentiation and/or depression in the isolated frog's toe muscle produced by naloxone (▲) and by quaternary naloxone (●), at various concentrations. Both compounds were tested on the same muscles and under identical experimental conditions. Each point is the mean ± S.E.M. from 8 muscles. When the S.E.M. is not shown it fell within the symbol. *Note:* The experimental data for naloxone presented in this figure and that presented in Table 2 were obtained from different muscles.

concentration of an opioid. Thus, in such an experiment (Fig. 14), the time required by a 'control' twitch response to methadone (10^{-4} M) to attain its maximal amplitude was initially determined. Next, the same concentration of methadone was added to the bath but the stimulus was interrupted for the above period of time. When the stimulation was resumed it was noted that the twitch response was equivalent in magnitude to the maximal response obtained earlier when the muscle was stimulated regularly once every 30 sec. In addition, no staircase phenomenon or treppe had influenced the results. This was shown by the observation that an interruption of stimulus for the same (or even greater) period of time under drug-free conditions caused no change in the twitch amplitude. Identical results were obtained with morphine (10^{-4} M). (N=2).

4.1.1.2 Maximum rates of rise/fall in tension:

Three opioids, viz., methadone (10^{-5}, or 5 x 10^{-5} M), morphine (10^{-4} M), and meperidine (10^{-4} M) were tested separately for their effects on the maximum rates of rise (MRR) and the maximum rates of fall (MRF) of the twitch tension. These characteristics of the twitch were determined by electrical differentiation of the twitch response as described earlier under 'Tension
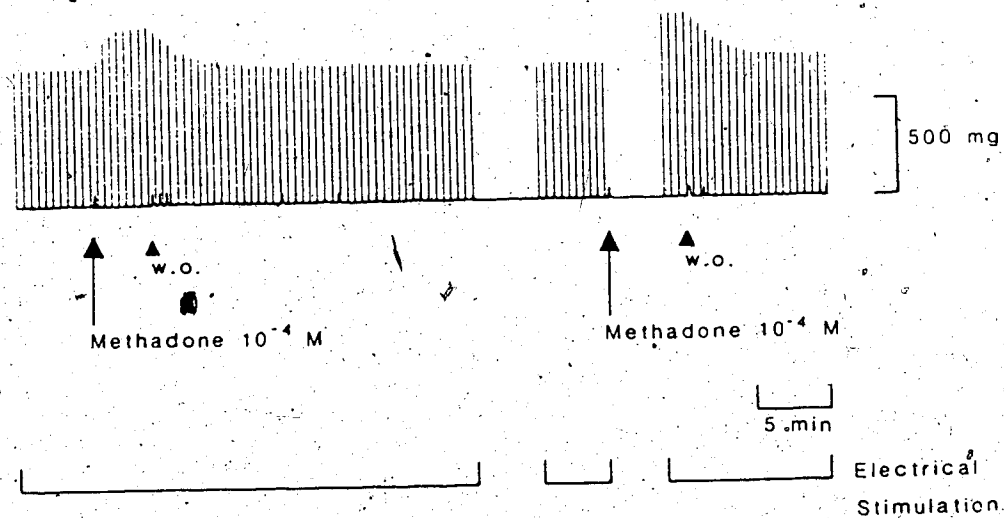
Figure 14. Lack of influence of repeated electrical
stimulation on either the time-course or the magnitude
of the twitch potentiation following exposure to
methadone (10^{-4} M). Horizontal lines below the record
show the periods during which the muscle was
electrically stimulated.

The above illustration was obtained from one
typical experiment of a set of 4 repetitions (i.e.,
N=4).

Recording' (see Ch.3.2.1.4). In all these experiments, the twitch responses and their first differentials were recorded simultaneously.

With all the opioids tested, an increase in the MRR and in the MRF of the twitch tension accompanied the twitch amplitude potentiation. This increase in the rates of change in tension was found to be dose-dependent and paralleled the dose-dependent increase in twitch amplitude (Table 4).

4.1.2 Summated Responses:

The effects of methadone (10^{-5} or 5×10^{-5} M), morphine (10^{-5} or 10^{-4} M), or meperidine (10^{-4} M) on muscle responses to closely spaced multiple electrical stimuli were studied.

The multiple stimuli consisting of trains of a discrete number of electrical pulses (2, 3, 5, 7 or 10) applied once every 30 sec to a muscle. Methadone (5×10^{-5} M) was the only concentration that was tested using trains of up to 7 electrical pulses; whereas methadone (10^{-5} M), morphine (10^{-5} or 10^{-4} M) or meperidine (10^{-4} M) were each tested only with 2 or 3 pulse trains. Maximally summated responses were recorded both, in the absence as well as in the presence of the opioids; the responses being obtained and identified as described earlier in the section labelled 'General Experimental Protocols' (Ch. 3.2.1.5). In this section, results have often been presented for an individual

TABLE 4.

Effect of opioids on various twitch parameters of the isolated toe muscle of frog.

Opioid, Concentration(M)	Twitch Parameters (% Control)		
	Tension	MRR	MRF
Methadone, 1×10^{-5}	126.64 ± 3.37	111.81 ± 2.60	120.88 ± 4.15
Methadone, 5×10^{-5}	144.04 ± 9.87	116.50 ± 2.86	126.66 ± 6.61
Morphine, 1×10^{-4}	123.09 ± 3.53	120.00 ± 4.90	125.06 ± 8.51
Meperidine, 1×10^{-4}	114.64 ± 1.30	110.47 ± 3.46	119.44 ± 5.89

Note : The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M. (n = 4).
MRR, maximal rate of rise of twitch tension;
MRF, maximal rate of fall of twitch tension.

representative experiment even though the experiment itself had been performed more than once. This was done with the sole purpose of illustrating the general trends observed in such experiments.

4.1.2.1 Amplitude:

Methadone (10^{-5} M) potentiated the amplitudes of the maximally summated responses to the multiple stimulation (Fig. 15). However, the percent increase in twitch size, compared with its 'control' without drug, decreased with an increase in the number of pulses in the train of electrical stimuli (Fig. 16 - amplitude). Similar results were obtained with methadone (5 x 10^{-5} M) (Fig. 17 - amplitude). This reduction was not a consequence of the muscle reaching its maximal contractile ability because the peak tensions developed by summated responses, in the presence of methadone, were less than that developed during the maximal tetanic response of the muscle. This was particularly true with summated responses to trains of 2 or 3 pulses. Generally, the peak tensions developed by maximal tetanic responses were 3 - 5 times those developed during single twitches.

Morphine, in a concentration that did not potentiate the amplitude of the single twitch, viz., 10^{-5} M, did not affect the heights of the responses to
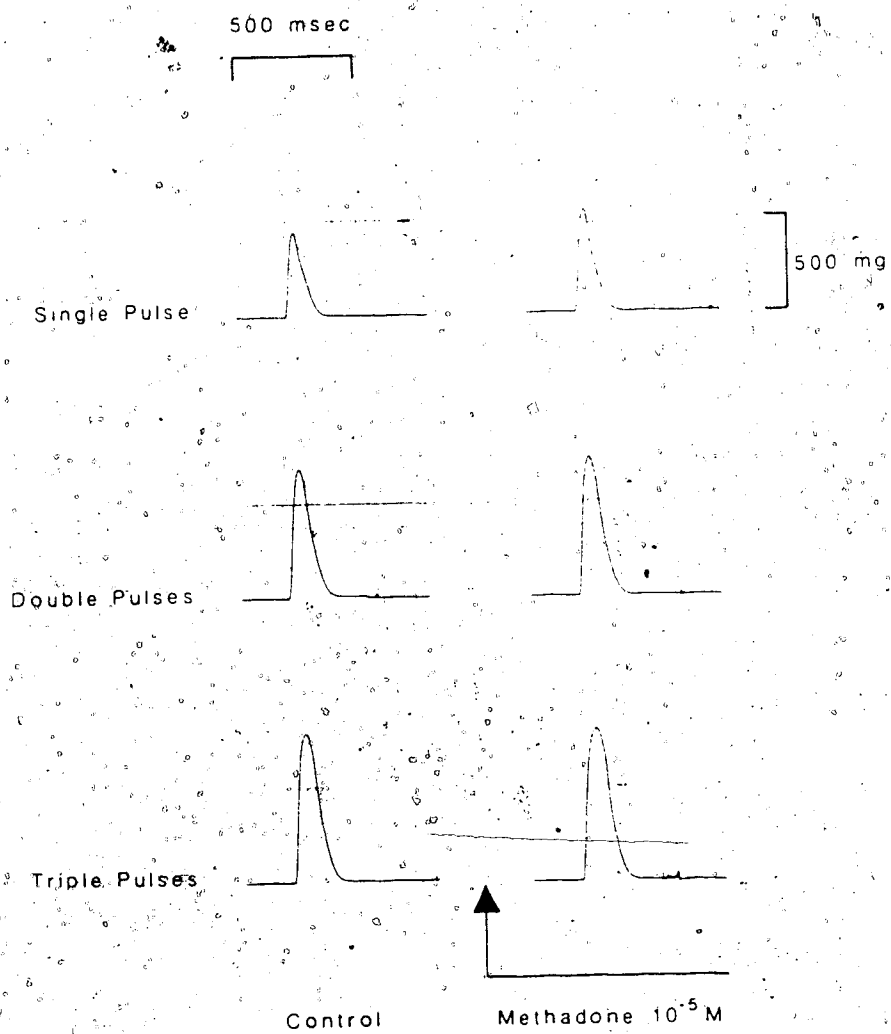
Figure 15. Effect of methadone (10^{-5} M) on the twitch and on fused responses produced by 2 or 3 pulses in a single toe muscle of the frog. Figure shows recordings obtained in one experiment from a set of 3 repetitions (i.e., N=3).
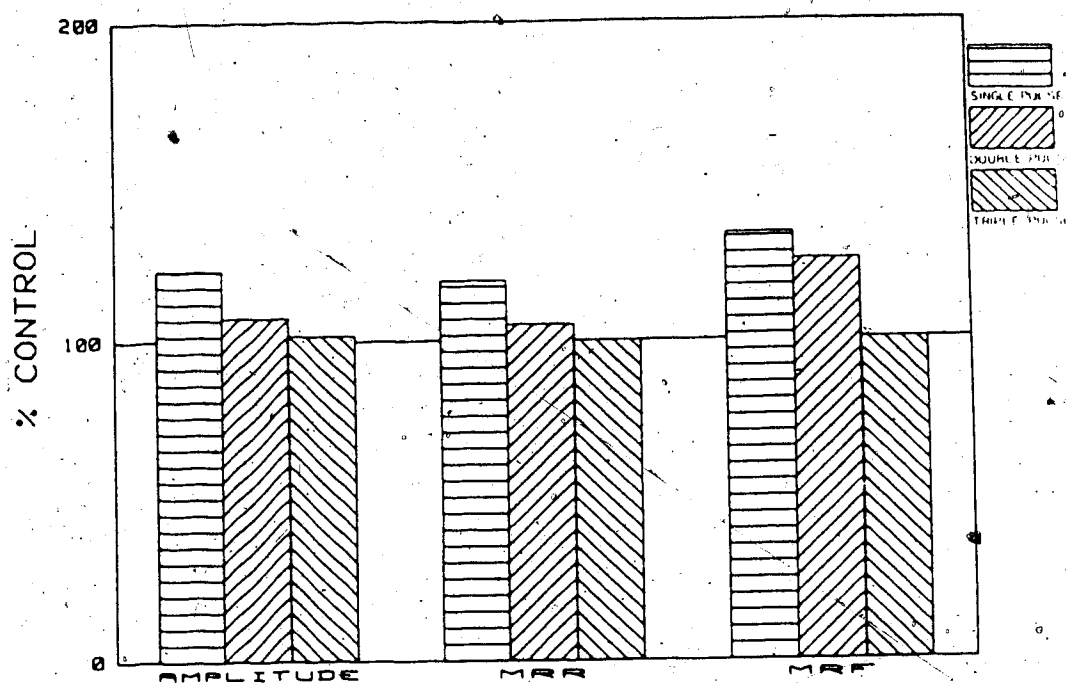
Figure 16. Decrease in the potentiation produced by methadone (10^{-5} M) as the number of pulses producing a fused response are increased. Single twitches and maximally summated responses evoked respectively by single and multiple (double or triple) electrical shocks were obtained both, in the absence and in the presence of methadone. The % control for the various twitch parameters, viz., amplitude, maximum rate of rise (MRR), and maximum rate of fall (MRF), were calculated using their respective without drug controls. The results presented were obtained from the recordings shown in Fig. 15. Thus these results were from one experiment of a set of 3 repetitions (i.e., N=3).
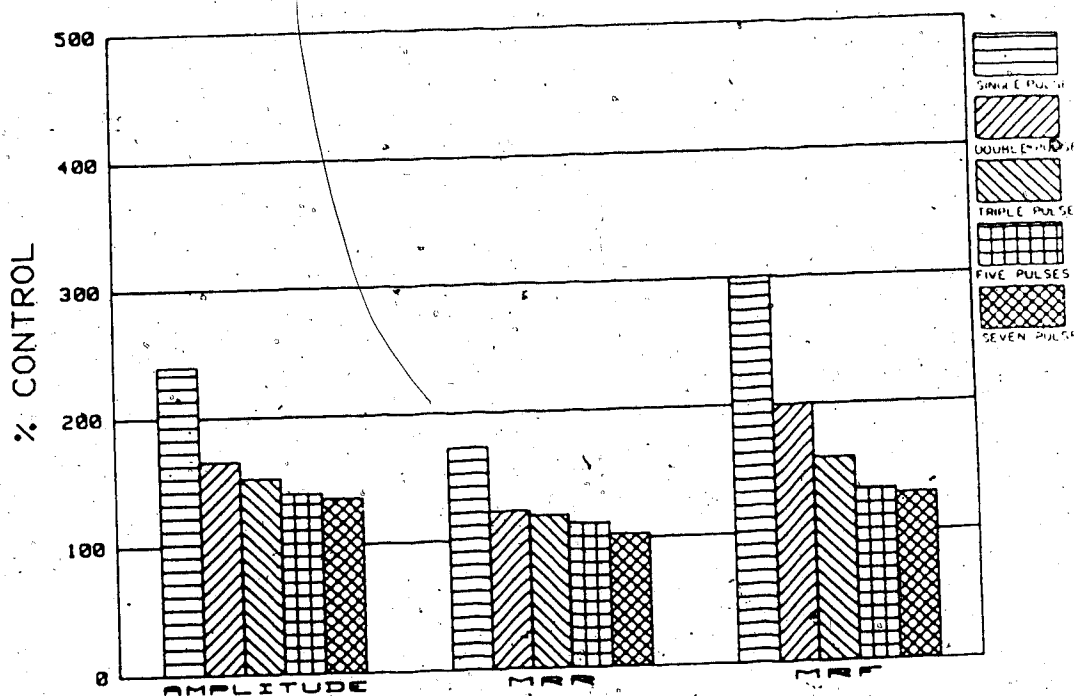
Figure 17. Decrease in the potentiation produced by methadone (5 x 10^{-5} M) as the number of pulses producing a fused response are increased. Single twitches and maximally summated responses evoked respectively by single and multiple (2-7) electrical shocks were obtained both, in the absence and in the presence of methadone. The % control for the various twitch parameters, viz., amplitude, maximum rate of rise (MRR), and maximum rate of fall (MRF), were calculated using their respective without drug controls. These results were obtained from one experiment of a set of 3 repetitions (i.e., N=3).

either the double or the triple pulses. A higher concentration of morphine, viz., 10^{-4} M, however, produced a pattern of response similar to that observed earlier with methadone (Fig. 18 - amplitude).

Interestingly, meperidine (10^{-4} M) which potentiated twitches to single stimuli, did not increase the size of summated responses, but on the contrary, these summated responses were smaller in magnitude than their 'control' responses (Fig. 19 - amplitude).

4.1.2.2 Maximum rates of rise/fall in tension:-

The maximum rate of rise (MRR) and the maximum rate of fall (MRF) in the tensions of the maximally summated 'control' responses (following trains of 2, 3, 5 or 7 electrical pulses) showed progressive increases in magnitudes in correspondence with the progressive increase in the twitch heights (Fig. 20).

The effects of opioids on the maximum rates of change in the tensions were qualitatively similar to their effects on the twitch amplitudes described earlier. In the presence of methadone (10^{-5} or 5 x 10^{-5} M), the MRR and the MRF of the maximally summated responses showed a progressive decrease in the percent increase of the parameter with an increase in the number of pulses per train of electrical stimuli (Fig.
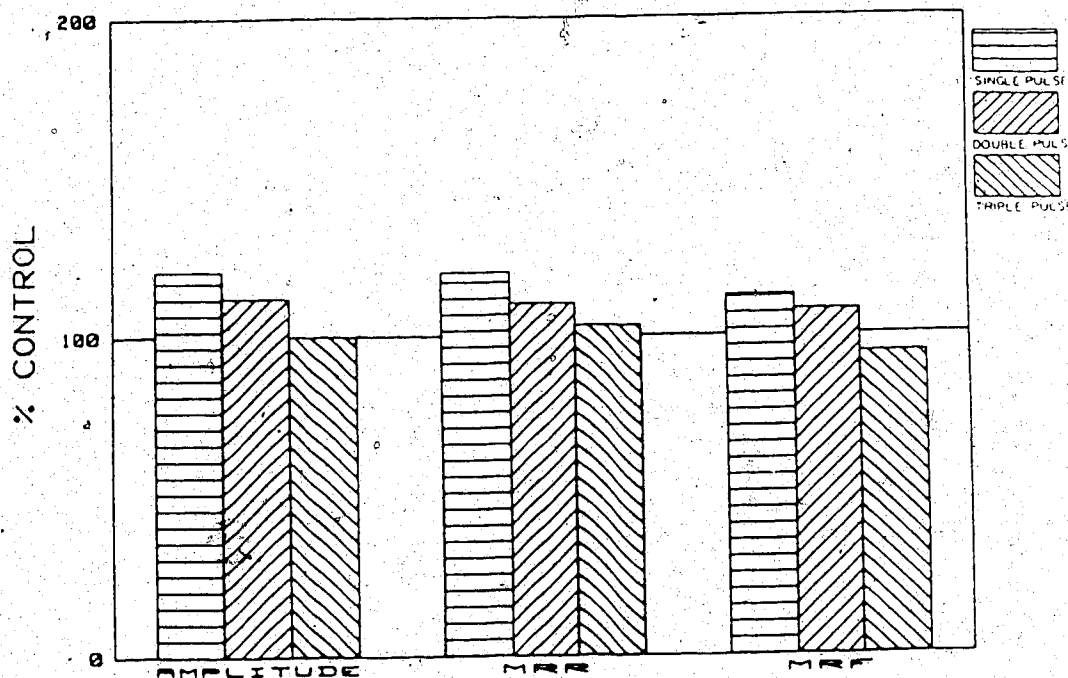
Figure 18. Decrease in the potentiation produced by morphine (10^{-4} M) as the number of pulses producing a fused response are increased . Single twitches and maximally summated responses evoked respectively by single and multiple (double or triple) electrical shocks were obtained both, in the absence and in the presence of morphine. Twitch responses and their 1st differentials were simultaneously recorded in each case. The % control for the various twitch-parameters, viz., amplitude, maximal rate of rise (MRR), and maximal rate of fall (MRF) were calculated using their respective without drug controls. These results were from one experiment of a set of 3 repetitions (i.e., N=3).
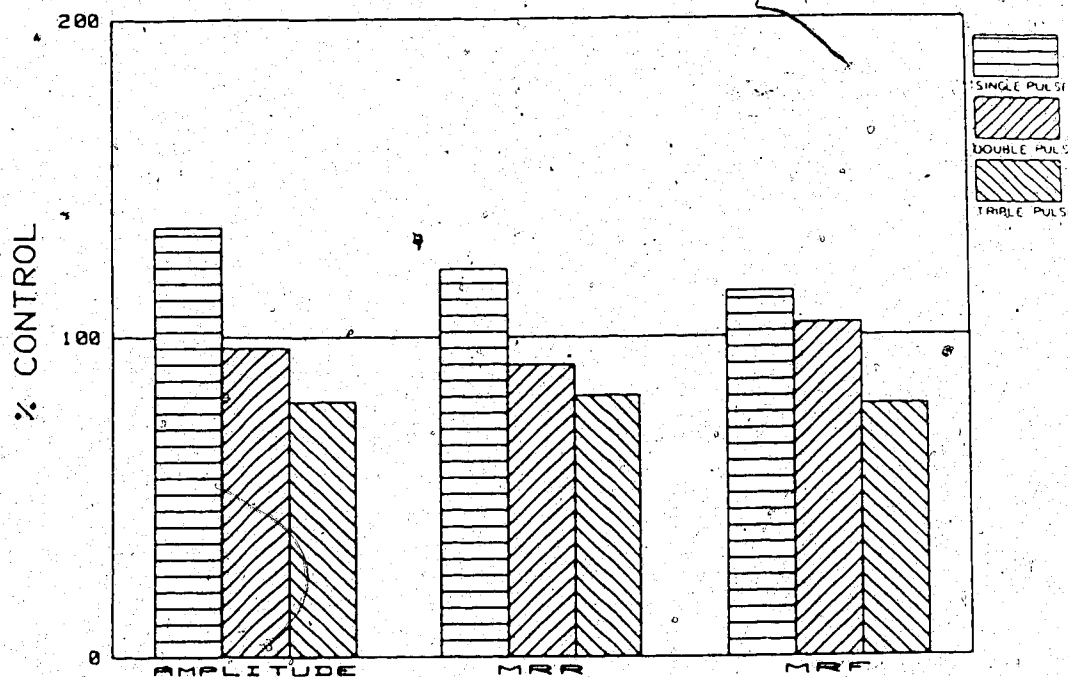
Figure 19. Progressive decrease in the size of the responses produced by meperidine (10^{-4} M) as the number of pulses producing a fused response is increased. Single twitches and maximally summated responses evoked respectively by single and multiple (double or triple) electrical shocks were obtained both, in the absence and in the presence of meperidine. The % control for the various twitch parameters, viz., amplitude, maximum rate of rise (MRR), and maximum rate of fall (MRF) were calculated using their respective without drug controls.
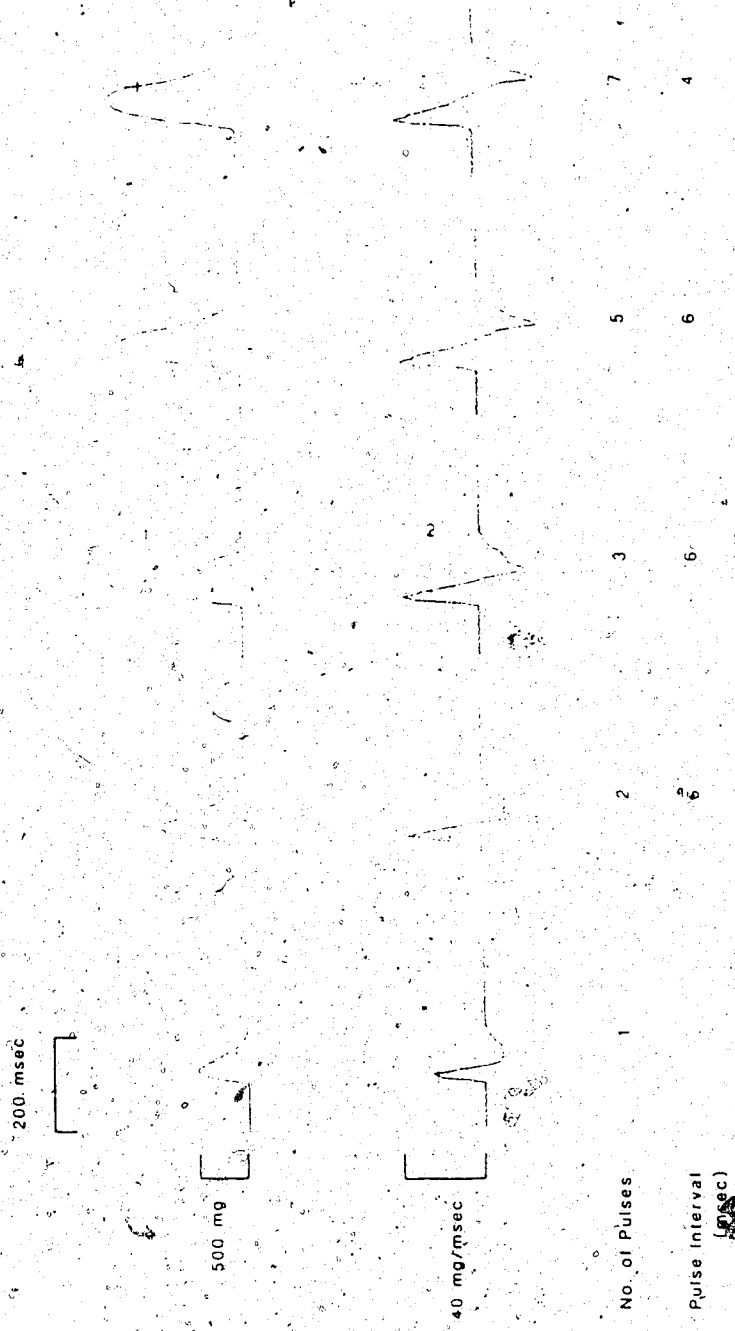
Figure 20. Progressive increase in the magnitudes of the maximal rate of rise and the maximal rate of fall of the maximally summated response as the number of pulses in a stimulus train is increased. Upper tracing, tension recording; lower tracing, 1st differential recording. This figure presents typical results from an individual experiment of a set of 3 repetitions (i.e. N=3).

16, 17).

Morphine (10^{-5} M) in a concentration that did not affect the response height also did not affect the rates of change in twitch tension; whereas a higher concentration, viz., 10^{-4} M produced changes in MRR and MRF similar to these observed earlier with methadone (Fig. 18).

With meperidine (10^{-4} M) there was no potentiation of the MRR or the MRF of the maximally summated responses as seen earlier with methadone or morphine. On the contrary, with meperidine there was only a progressive decrease in the MRR or the MRF of the response with an increase in the number of pulses per train of stimuli (Fig. 19).

4.1.2.3 Time to peak tension:

The time to peak tension was determined for a single twitch and for maximally summated responses following trains of 2, 3, 5, 7 or 10 electrical pulses.

A plot of peak tension vs time to peak tension indicated that the progressive increase in the peak tension generated by a summated response was accompanied by a corresponding increase in the time to peak tension (Fig. 21). The relationship appeared to be hyperbolic in nature and showed a tendency to plateau. Methadone (5 x 10^{-5} M) did not affect the
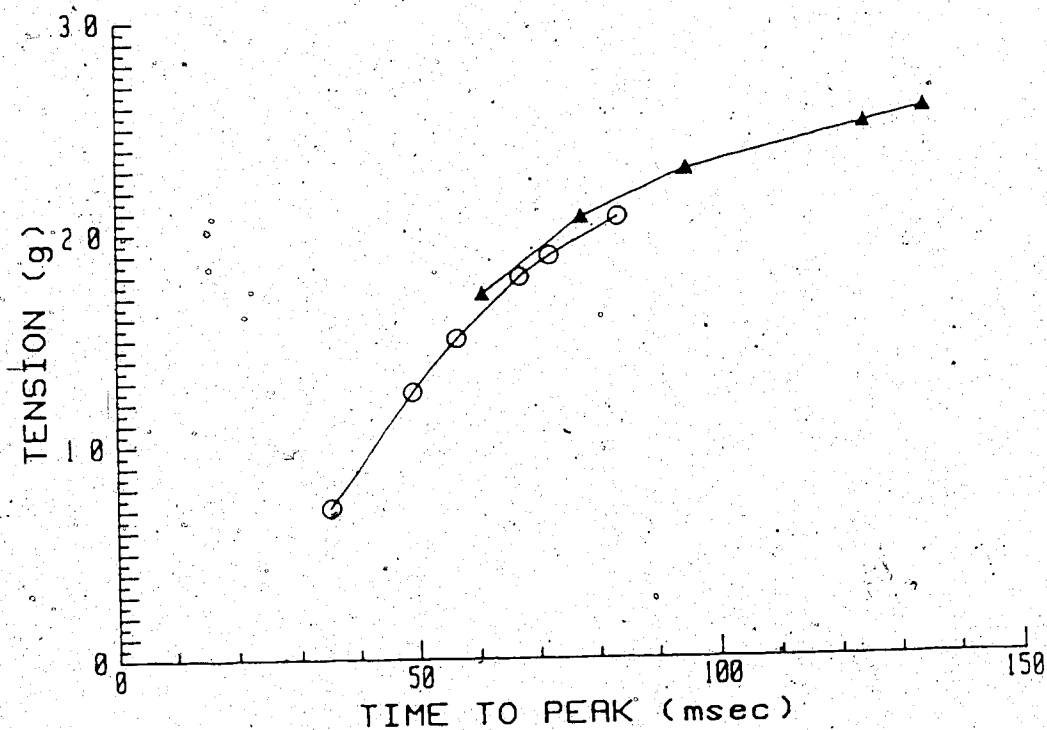
Figure 21. Effect of methadone (5 x 10^{-5} M) on the time to peak tension of the twitches and the maximally summated responses elicited by 1, 2, 3, 5, 7, and 10 electrical pulses. Twitch responses were obtained in the absence (O, control) and in the presence of methadone (▲, test). For each curve the tension increased with the number of pulses. The results presented were obtained from an individual experiment from a set of 3 repetitions (i.e., N=3)

relationship existing between the peak tension and the time to peak tension of the twitch; it only moved it to higher values of tension (Fig. 21).

4.1.2.4 Fusion Frequency:

Twitch responses were recorded to trains of dual or triple electrical pulses. The responses were obtained using pulse intervals ranging from 0.5 to 100 msec. The twitches obtained with small pulse intervals, viz., 0.5 or 1 msec were usually identical with the twitch following a single stimulus but as the pulse intervals were increased the summation was observed which increased until the maximally summated response was obtained. Any further increase in the pulse interval (or decrease in the frequency of stimulation) caused the response to the multiple stimuli to be unfused resulting in tension records showing multiple peaks in correspondence with the multiple stimuli (Fig. 4). The reciprocal of the pulse interval at which the response is completely fused is the critical fusion frequency (or fusion frequency).

Methadone (10^{-5} M) which produced a slight twitch potentiation did not affect the fusion frequency whereas a higher twitch potentiating concentration of the drug, viz., 5×10^{-5} M shifted the pulse intervals at which the responses were completely fused, viz.,

from 0.5 to 10 msec to 0.5 to 30 or 40 msec (Figs. 22 - 23),

Morphine (10^{-5} or 10^{-4} M) or meperidine (10^{-4} M) showed no such effect on the fusion frequency.

4.1.3 Tetanus:

Single twitches and maximal tetanic responses were obtained and recorded as described in the section labelled 'Stimulation Parameters', (Ch. 3.2.1.3). These responses were obtained in the absence and then in the presence of an opioid and the results were compared. In the latter case, the tetanic stimulation was started at a time when the twitch potentiating effect of the opioid had peaked.

Morphine, in concentrations that did not potentiate the twitch (viz. 10^{-5} M), produced no change in the maximal tetanic response of the muscle. The opioid in higher concentrations, viz., 5×10^{-5} or 10^{-4} M produced typical twitch potentiations but it did not potentiate the peak tension of the maximal tetanus. On the contrary, in the presence of the opioid, the plateau of the tetanic response was not sustained, but declined in an irregular fashion during stimulation. The single twitches immediately following the tetanic response were however unaffected, i.e., they continued to exhibit their opioid potentiated tension (Fig. 24).
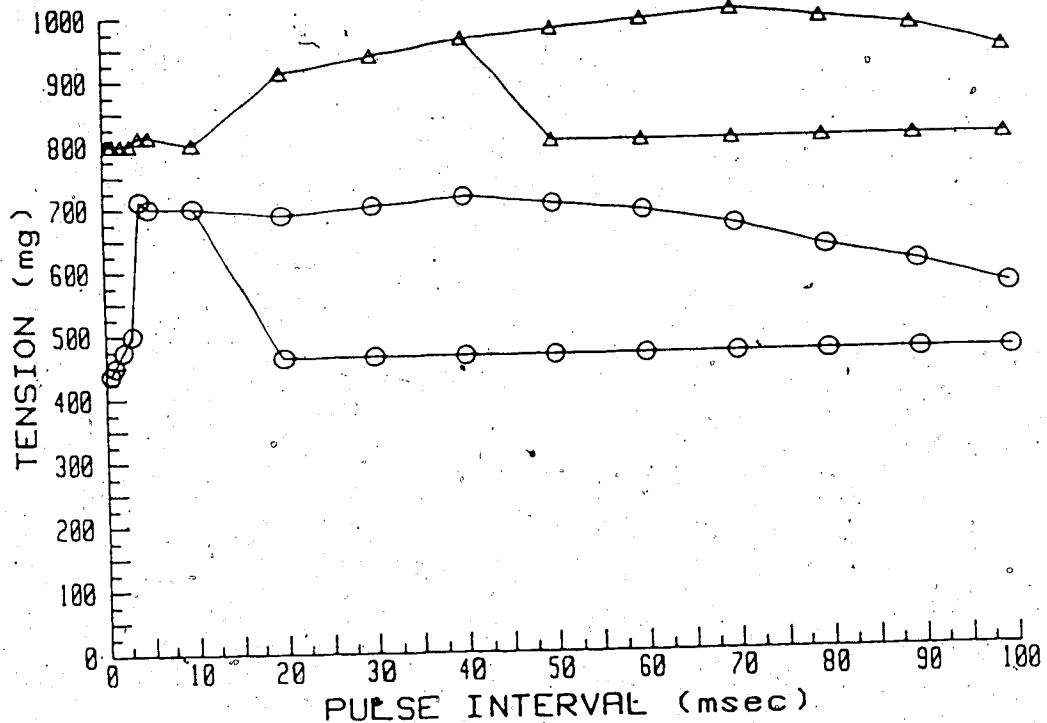
Figure 22. Effect of a high concentration of methadone (5 x
10⁻⁵ M) on the critical fusion frequency of the
response of the frog's toe muscle to closely spaced
double electrical pulses. Twitch responses of the
muscle to trains of double pulses (pulse interval, 0.5
- 100 msec) were obtained both, in the absence (O,
control), and in the presence (Δ, test) of methadone.
Pulse intervals >10 msec and >40 msec produced unfused
responses under control and under test conditions,
respectively. The height of each of the 2 peaks of the
unfused response (control or test) at any pulse
interval is represented by the two points plotted
against that pulse interval; the 1st and 2nd peak being
represented in order by the lower and the higher point.
These results were obtained from one experiment of a
set of 3 repetitions (i.e., N=3).
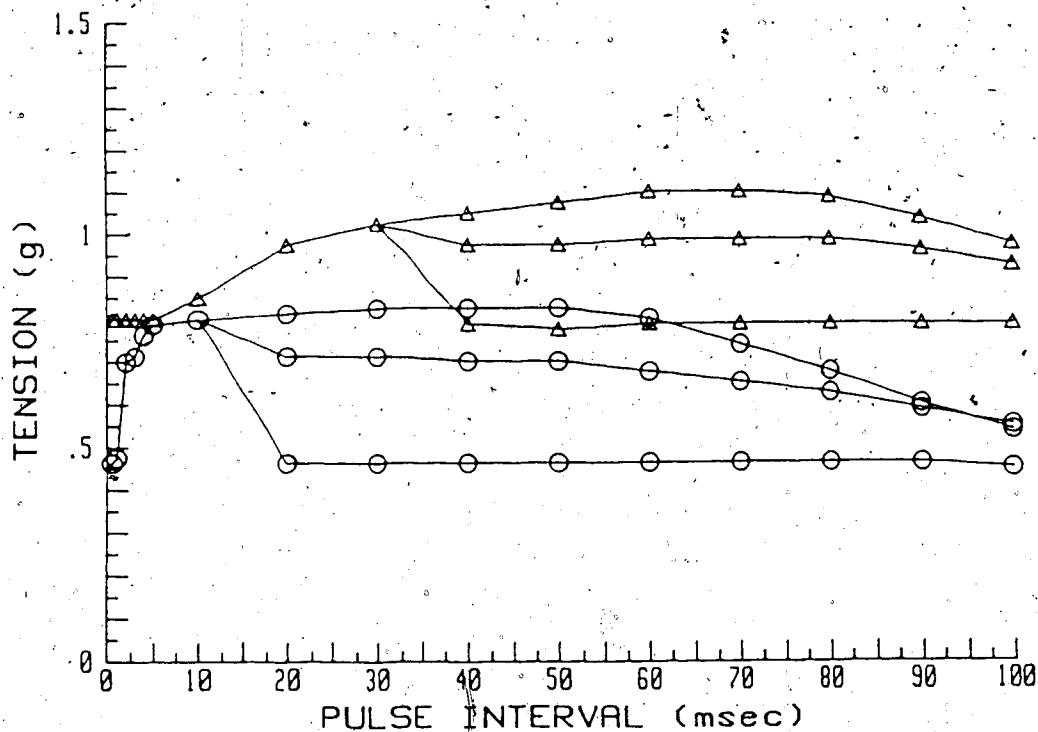
Figure 23. Effect of a high concentration of methadone (5 x
10⁻⁵ M) on the critical fusion frequency of the
response of the frog's toe muscle to closely spaced
triple electrical pulses. Twitch responses of the
muscle to trains of triple pulses (pulse interval, 0.5
- 100 msec) were obtained both, in the absence (O,
control), and in the presence (Δ, test) of methadone (5
x 10⁻⁵ M). Pulse intervals >10 msec and >30 msec
produced unfused responses under control and test
conditions, respectively. The height of each of the 3
peaks of the unfused response (control or test) at any
particular pulse interval is indicated by the 3 points
plotted against that pulse interval; the 1st, 2nd and
3rd peak being represented in order by the lowest, the
intermediate, and the highest point. These results
were obtained from one experiment of a set of 3
repetitions (i.e., N=3).

Control

Morphine 0.1 mM

w.o.

1.0 g

5 Min   1 Sec
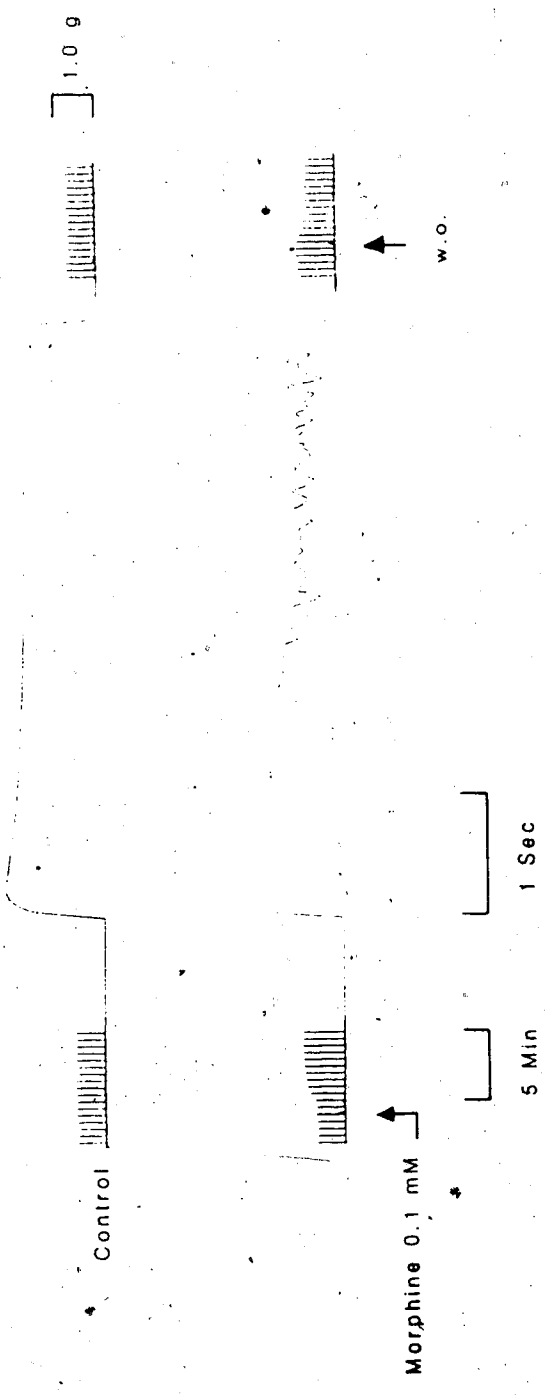
Figure 24. Comparison of the effects of morphine (10^{-4} M) on the twitch and the tetanus of an isolated frog's toe muscle. The twitch responses were elicited by single electrical pulses once every 30 sec. The tetanic responses were elicited by trains of 60 pulses/sec applied for 5 sec to the muscle. The above illustration was obtained from one experiment of a series of 4 repetitions (i.e. N=4).

Identical results were obtained with the other opioids
tested, viz., methadone (10^{-4} M) or meperidine (10^{-4} M).

4.2 INVESTIGATION OF THE POSSIBLE INVOLVEMENT OF A STEREOSPECIFIC OPIOID RECEPTOR IN THE TWITCH POTENTIATION AND DEPRESSION PRODUCED BY OPIOIDS:

4.2.1 Twitch Potentiation:

4.2.1.1 Studies with Opioid Antagonists

To test whether the twitch potentiations were mediated via a stereospecific opioid receptor mechanism, naloxone was used as a selective opioid antagonist. The muscles were exposed to the antagonists for varying periods of time ranging from 10 min to 45 min before being tested with the combination of the agonist and the antagonist.

Naloxone (5×10^{-7} M) did not affect the twitch potentiation produced by 5×10^{-5} M methadone (Table 5). Higher concentrations of naloxone, viz., 5×10^{-6} M or 2×10^{-5} M, did cause a small antagonism of methadone effects (Fig. 25); however, such concentrations of the antagonist when tested alone caused depressions of the 'control' twitch responses (Table 2). Naloxone, 4×10^{-5} M, a concentration that had negligible effects on the 'control' twitch response, did not antagonize the twitch potentiating effects of methadone (4×10^{-5} M or 8×10^{-5} M) (Table 5). Further increases in naloxone concentrations

TABLE 5.

Effect of naloxone on the twitch potentiation produced by methadone in the isolated toe muscle of frog.

Naloxone Concentration (M)	Methadone Concentration (M)	n	% Twitch Potentiation		
			Naloxone	Methadone	Methadone + Naloxone
5×10^{-7}	5×10^{-5}	4	100.0 ± 0.0	126.6 ± 2.1	131.5 ± 4.8
4×10^{-5}	4×10^{-5}	6	98.2 ± 1.2	122.3 ± 1.6	121.1 ± 3.8
4×10^{-5}	8×10^{-5}	6	96.6 ± 1.6	141.2 ± 3.4	141.9 ± 4.7
8×10^{-5}	4×10^{-5}	6	106.4 ± 1.1	123.4 ± 1.9	137.5 ± 2.1
8×10^{-5}	8×10^{-5}	6	112.4 ± 1.3	147.9 ± 3.0	157.4 ± 3.2

Note : The muscles were exposed to the antagonist for 45 min before testing the combination of the agonist and the antagonist.
The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M.
The calculated t-values were not significant.
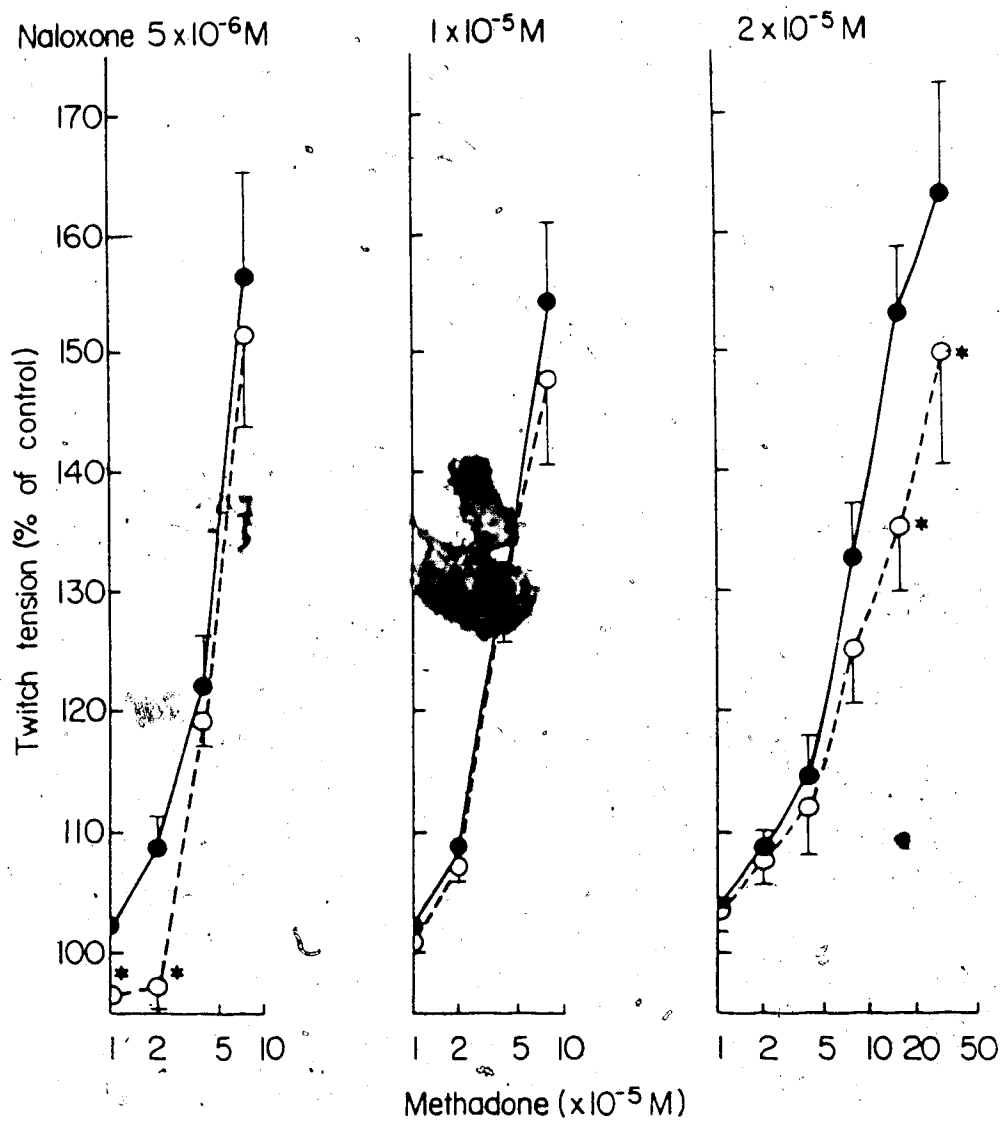
Figure 25. Effect of naloxone on the twitch potentiation produced by methadone in the isolated frog's toe muscle. ●---●, methadone alone; O---O, methadone plus naloxone. Data are expressed means ± S.E.M. from experiments with six muscle preparations. *, means with and without naloxone significantly different at p=0.05. A paired t-test was conducted.

revealed a twitch potentiating effect of the antagonist itself (Table 2, 5; Fig. 13). Thus, when 8 x 10^{-5} M naloxone was tested for antagonism of methadone (4 x 10^{-5} or 8 x 10^{-5} M) effects, the agonist response was not inhibited, on the contrary it was further enhanced (Table 5) apparently due to a summation of the twitch potentiating effects of the two compounds. Additionally, the increases in twitch tensions caused by methadone, meperidine, (-)ketazocine, and (-)ethyl ketazocine were not antagonized by various concentrations of known opioid antagonists, naloxone, naltrexone, phenoxybenzamine or Mr2096 even with exposures to the antagonists up to 45 min (Table 6). Naloxone (10^{-5} M) was also unable to antagonize the twitch potentiating effects of met-enkephalin (10^{-6} – 10^{-3} M) or leu-enkephalin (10^{-7} – 10^{-3} M) when tested in four experiments using eight toe muscles.

4.2.1.2 Studies with Opioid Stereoisomers

It was observed that levorphanol, a potent opioid which has the D(-) configuration and dextrorphan, the analgesically inactive L(+) isomer, exhibited no significant differences in their relative potencies in potentiating the twitch (Fig. 11). On the other hand (+)morphine, the analgesically inactive isomer of morphine, required a concentration of 10^{-3} M to produce

TABLE 6.

Effects of opioid antagonists on the twitch potentiation produced by opioid agonists in the toe muscle of frog.

Agonist, concentration (M)	Twitch potentiation by agonist (% control)	Antagonist, concentration (M)	Exposure time (to antagonist) (Min)	Twitch potentiation by agonist in presence of antagonist (% control)
Ethyl Ketazocine (-), 10^{-4} *	111.3 ± 5.1	Naloxone, 2×10^{-5}	45	115.4 ± 2.9
Ketazocine (-), 10^{-4} **	134.2 ± 9.7	Naloxone, 10^{-9}	10	135.7 ± 3.7
			45	137.7 ± 1.7
	134.2 ± 9.7	Naloxone, 10^{-7}	10	136.9 ± 6.1
			45	131.3 ± 4.4
	134.2 ± 9.7	Naloxone, 10^{-5}	10	136.9 ± 6.1
			45	136.9 ± 2.4
	134.2 ± 9.7	Naloxone, 2×10^{-5}	10	128.6 ± 7.2
			45	128.6 ± 7.2
Meperidine, 4×10^{-4} **	163.7 ± 9.3	Naltrexone, 10^{-9}	10	160.6 ± 7.8
			25	155.7 ± 8.1
			45	151.8 ± 9.0
	156.8 ± 1.2	Naltrexone, 10^{-7}	10	154.9 ± 9.9
			25	158.8 ± 8.8
			45	162.8 ± 7.8
	212.9 ± 3.9	Naltrexone, 10^{-5}	10	217.9 ± 35.9
			45	214.3 ± 35.7
Methadone, 8×10^{-5} *	145.6 ± 2.7	Phenoxybenzamine, 10^{-7}	45	157.0 ± 5.6
	149.7 ± 2.7	Phenoxybenzamine, 10^{-6}	45	140.1 ± 5.5
Methadone, 4×10^{-4} **	140.8 ± 15.5	Mr2096, 10^{-9}	10	142.1 ± 24.7
			45	144.2 ± 22.5
	132.4 ± 8.0	Mr2096, 10^{-7}	10	135.7 ± 8.9
			45	140.3 ± 8.2
	132.3 ± 8.0	Mr2096, 10^{-5}	10	138.4 ± 9.7
			45	136.3 ± 7.7
	132.4 ± 8.0	Mr2096, 10^{-4}	10	151.3 ± 10.7
			45	160.3 ± 12.2

Note: The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M. (n=6).
* The calculated t-value was not significant ($p < 0.05$).
** F-value calculated from the ANOVA was not significant ($p < 0.05$).

twitch potentiation as compared to (±) morphine which produced a threshold effect at 10^{-5} M (Table 2).

4.2.2 Twitch Depression:

The depression of twitch height following prolonged exposures to high concentrations of opioid agonists was investigated for an involvement of a stereospecific opioid receptor.

Naloxone (10^{-7} - 10^{-5} M), a specific opioid antagonist, did not affect the time taken by morphine (10^{-3} M) or fentanyl (10^{-3} M) to produce a 50% inhibition of the 'control' twitch response even though the muscle was equilibrated with the antagonist for 45 min prior to testing for an antagonism of the agonists' effects (Table 7).

Effect of naloxone on twitch depression produced by high concentrations of opioid drugs in the isolated toe muscle of frog.

TABLE 7.

Agonist, concentration (M)	Time required by agonist to produce 50% depression of twitch height (Min)	Naloxone concentration (M)	Time taken by the combination of agonist and naloxone to produce 50% depression of twitch height (Min)
Morphine, 10^{-3} *	41.0 ± 8.5	10^{-4}	49.5 ± 7.3
Fentanyl, 10^{-3} **	3.5 ± 1.2	10^{-9}	2.7 ± 0.3
		10^{-7}	2.7 ± 0.4
		10^{-5}	2.2 ± 0.4

NOTE: Naloxone was exposed to the tissue for 45 min before testing for antagonism of the depression of twitch height.
Means ± S.E.M. (n=4)
* The calculated t-value was not significant ($p < 0.05$).
** F-value calculated from the ANOVA was not significant ($p < 0.05$).

4.3 INVESTIGATION OF THE MECHANISM UNDERLYING THE TWITCH POTENTIATING EFFECTS OF OPIOIDS.

4.3.1 Opioid Effects on the Electrical Properties of the Frog Skeletal Muscle Fibre Membrane

In all electrophysiological studies extracellular stimulation and intracellular microelectrode recording techniques were used. Typical recordings made with intracellular microelectrodes are shown in Fig. 7. Tracings such as these were used to obtain values of the electrical parameters of interest.

Under the experimental conditions used in this laboratory the electrical properties of the frog sartorius muscle placed in Ringer's solution were reported to remain unchanged up to 6 hr [262]. Since most of our experiments lasted only 30 min and none lasted longer than 6 hr, we assumed that the experimental conditions employed in this study do not by themselves alter any of the electrical properties of the muscle fibre membrane.

Dextrorphan: The effects of dextrorphan on the electrical parameters of the frog sartorius muscle were studied using concentrations (10^{-5}, 3×10^{-5}, 10^{-4} or 3×10^{-4} M) of the drug that produced twitch potentiations in the frog toe muscle that ranged from about threshold to supramaximal (Fig. 11).

The resting membrane potential of the muscle was unaffected by the opioid when tested at 5, 10, 20 and 30 min following drug exposure. The height of the action potential too was unaffected by 10^{-5}M, 3×10^{-5}M, or 10^{-4}M dextrorphan when tested up to 30 min following drug exposure but was depressed by higher drug concentrations. The maximum rates of rise/fall of the action potential were depressed by higher concentrations of the compound and concurrently, the duration of the action potential (measured at -40 mV) was enhanced (Table 8).

Thus, low concentrations of dextrorphan, viz., 10^{-5} or 3×10^{-5} M did not produce any change in the electrical parameters of the frog skeletal muscle fibre membrane, within 10 min of drug exposure (Table 8), although under similar conditions they produced significant twitch potentiations in the frog toe muscle (Fig. 11). Higher concentrations of the opioid, however, did prolong the action potential duration by 5 min of drug exposure and at the same time depressed its amplitude and its maximum rates of rise and fall. As shown in Fig. 26, the increase in the twitch height in presence of the opioid is clearly unrelated to changes in the action potential duration.

Naloxone: Naloxone, an opioid antagonist, which also potentiated the twitch of frog toe muscle (Table 2) was tested for effects on the electrical properties of the frog sartorius muscle fibre membrane. For this purpose, the drug

TABLE 8.

Effect of dextrorphan on the electrical properties of the frog's sartorius muscle.

Dextrorphan Concentration (M)	Time in Dextrorphan (Min)	Resting Potential (mV)	Action Potential			
			Amplitude (mV)	Maximum Rate of Rise (V/sec)	Maximum Rate of Fall (V/sec)	Duration (at -40 mV) (msec)
1×10^{-5}	0	87.19 ± 3.38	117.56 ± 5.13	418.65 ± 8.24	145.31 ± 8.10	1.017 ± 0.089
	5	86.05 ± 3.45	113.41 ± 6.00	373.40 ± 14.08	133.82 ± 9.03	1.041 ± 0.124
	10	90.00 ± 3.73	119.37 ± 5.34	419.29 ± 4.50	134.01 ± 8.90	1.051 ± 0.116
	20	89.01 ± 2.42	116.27 ± 5.12	403.39 ± 9.93	122.48 ± 9.38	1.108 ± 0.114
	30	88.19 ± 3.61	117.94 ± 7.16	418.62 ± 16.51	123.55 ± 13.06	1.161 ± 0.143
3×10^{-5}	0	88.33 ± 2.61	113.23 ± 2.41	382.97 ± 39.91	124.22 ± 3.43	1.081 ± 0.076
	5	87.41 ± 3.84	110.20 ± 2.46	388.12 ± 31.80	115.17 ± 8.35	1.075 ± 0.063
	10	86.61 ± 3.34	106.56 ± 5.09	368.57 ± 45.71	103.83 ± 10.91	1.144 ± 0.063
	20	88.37 ± 3.80	108.71 ± 1.87	361.98 ± 31.65	95.78 ± 6.26	1.254 ± 0.106
	30	86.82 ± 3.50	111.58 ± 5.65	395.17 ± 41.88	92.41 ± 2.29	1.353 ± 0.097
1×10^{-4}	0	83.90 ± 2.54	111.20 ± 6.13	418.10 ± 23.22	138.40 ± 9.32	0.930 ± 0.065
	5	81.80 ± 3.61	107.30 ± 4.56	419.20 ± 18.36	98.30 ± 8.65	1.321 ± 0.131
	10	82.80 ± 2.98	104.50 ± 6.01	374.60 ± 20.56	78.51 ± 8.43*	1.800 ± 0.101*
	20	81.00 ± 3.21	97.40 ± 5.32	326.20 ± 23.43*	49.02 ± 7.64*	2.550 ± 0.211*
	30	81.80 ± 2.56	96.80 ± 6.01	300.30 ± 20.36*	39.40 ± 9.32*	3.230 ± 0.121*
3×10^{-4}	0	87.50 ± 1.64	113.70 ± 5.95	350.00 ± 12.35	143.61 ± 10.11	0.950 ± 0.071
	5	92.20 ± 3.21	101.40 ± 3.26	266.70 ± 24.38*	46.32 ± 5.36*	2.180 ± 0.100*
	10	90.80 ± 3.45	100.00 ± 1.08*	258.50 ± 25.36*	34.41 ± 4.38*	2.991 ± 0.131*
	20	85.80 ± 3.23	95.90 ± 2.36*	227.10 ± 20.31*	15.80 ± 6.35*	4.210 ± 0.162*
	30	86.90 ± 3.01	85.40 ± 3.27*	149.20 ± 18.34*	11.00 ± 7.32*	4.500 ± 0.201*

Note : Means ± S.E.M. (n = 3).
* Significant differences from control values are as indicated: ($p < 0.05$). ANOVA followed by Newman Keuls multiple range test applied.
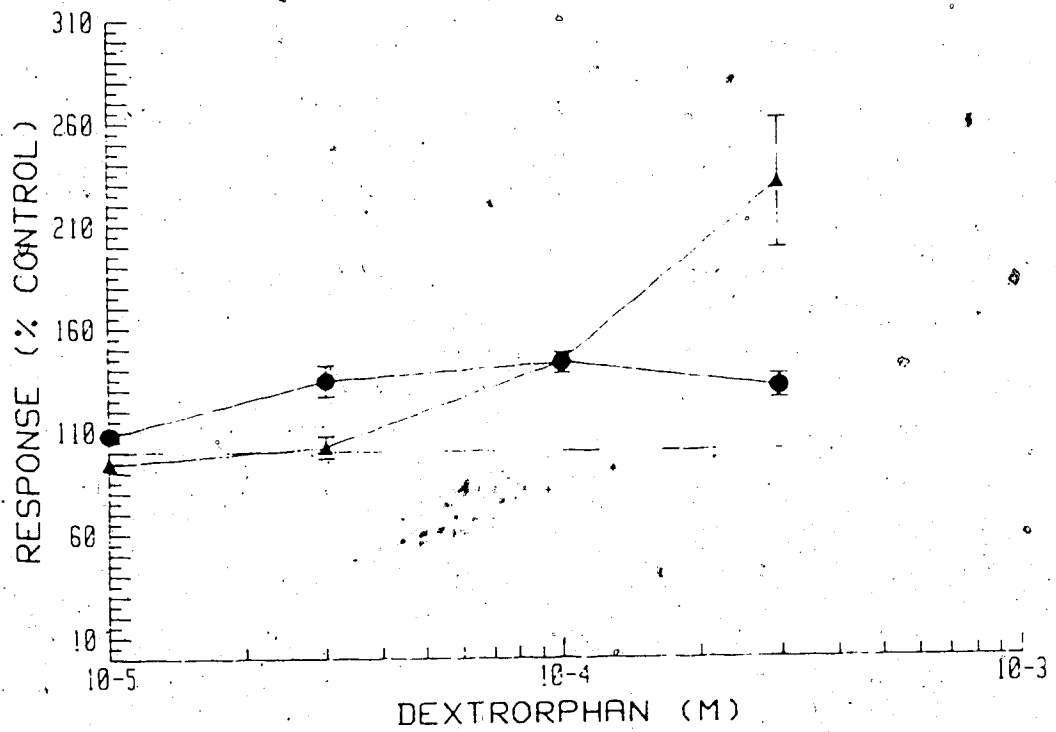
Figure 26. Effects of various dextrorphan concentrations
(10^{-5}, 3×10^{-5}, 10^{-4} and 3×10^{-4} M) on the action
potential duration (measured at -40 mV) and on twitch
height. ▲, action potential duration (n=3), data used
from Table 8; ●, twitch height (n=6), data used from
Fig. 11. Means ± S.E.M.

was employed in various concentrations (10^{-4}, 5 X 10^{-4} or 10^{-3} M); the lowest concentration being that which produced a small but significant twitch potentiation.

The resting membrane potential of the muscle was unaffected by the opioid antagonist. The amplitude of the action potential was depressed only with 10^{-3} M naloxone, the highest concentration tested. This was also the case for the maximum rate of rise of the action potential. In contrast, the maximum rate of fall of the action potential was more sensitive to naloxone. The duration of the action potential too was unaffected by low concentrations of the drug but was significantly prolonged by higher concentrations (Table 9).

Thus, the results with naloxone resemble those obtained with dextrorphan, in that the concentrations of either drug which produced small but significant twitch potentiations did not alter any of the measured electrical properties of the muscle fibre membrane within the time period required for the twitch-potentiating effect to develop fully.

Etorphine: The one opioid tested which lacked a twitch potentiating ability was tested for its effects on the electrical properties of the sartorius muscle. It was used in concentrations ranging from 10^{-5} to 10^{-3} M, which therefore included the concentrations that either produced no effect or only a depressant effect on the twitch (Fig. 12).

TABLE 9.

Effect of naloxone on the electrical properties of the frog's sartorius muscle.

Naloxone Concentration (M)	Time in Naloxone (Min)	Resting Potential (mV)	Action Potential			
			Amplitude (mV)	Maximum Rate of Rise (V/sec)	Maximum Rate of Fall (V/sec)	Duration at -40 mV (ms)
1 x 10⁻⁴	0	81.22 ± 4.46	109.39 ± 5.01	403.35 ± 29.89	142.23 ± 7.16	1.059 ± 0.063
	5	80.29 ± 3.57	104.41 ± 3.99	383.33 ± 7.37	137.59 ± 5.30	1.010 ± 0.043
	10	81.52 ± 3.79	107.53 ± 4.69	390.36 ± 18.48	135.23 ± 5.85	1.060 ± 0.039
	20	82.65 ± 3.18	108.05 ± 3.18	385.11 ± 11.65	128.87 ± 5.79	-1.107 ± 0.080
	30	81.64 ± 2.12	103.12 ± 3.08	340.01 ± 14.05	114.86 ± 7.36	1.197 ± 0.074
5 x 10⁻⁴	0	84.21 ± 4.14	114.84 ± 5.99	368.17 ± 55.38	124.22 ± 17.18	1.229 ± 0.160
	5	89.24 ± 3.07	115.33 ± 4.74	397.62 ± 28.05	110.66 ± 9.64	1.189 ± 0.114
	10	89.21 ± 2.80	110.78 ± 5.21	360.71 ± 32.27	90.73 ± 10.89	1.371 ± 0.154
	20	88.72 ± 2.91	109.62 ± 5.68	329.72 ± 39.72	77.47 ± 10.58	1.605 ± 0.181
	30	88.57 ± 3.33	112.80 ± 4.66	338.73 ± 26.00	69.50 ± 7.23*	1.695 ± 0.146
1 x 10⁻³	0	85.56 ± 0.90	115.46 ± 1.61	410.37 ± 15.81	124.42 ± 11.21	1.163 ± 0.051
	5	85.68 ± 0.95	104.18 ± 4.73	319.78 ± 29.74*	74.07 ± 10.89*	1.659 ± 0.212
	10	87.54 ± 1.36	100.50 ± 3.84	285.38 ± 20.72*	58.31 ± 7.13*	1.849 ± 0.255
	20	82.44 ± 2.14	88.59 ± 4.77*	200.21 ± 29.81*	42.47 ± 9.48*	2.266 ± 0.172*
	30	83.64 ± 0.38	90.10 ± 6.33*	203.45 ± 34.09*	38.29 ± 4.56*	2.425 ± 0.160*

Note : Means ± S.E.M. (n=3).
* Significant differences from control values are as indicated: (p < 0.05). ANOVA followed by Newman Keuls multiple range test applied.

Etorphine had no effect on the resting membrane potential of the muscle. The height of the action potential was not much affected by 10^{-5} or 10^{-4} M etorphine but it was depressed by 10^{-3} M. The maximum rate of rise of the action potential was not reduced by 10^{-5} M but was depressed by the higher drug concentrations. The maximum rate of fall of the action potential was decreased by 10^{-4} M or 10^{-3} M etorphine whereas the action potential duration was increased by 10^{-3} M etorphine (Table 10).

Thus, etorphine (10^{-3} M), too produced inhibitions in the action potential amplitude, and in its maximum rates of rise and fall and caused increments in the duration of the action potential. These changes were similar to those produced by dextrorphan or naloxone at concentrations that potentiated the twitch.

4.3.2 Interaction between Opioids and Na^+:

The influence of reduced extracellular Na^+ concentrations on opioid-effects on the twitch as well as on the electrical properties of the frog skeletal muscle fibre membrane, was studied. In all these experiments, reductions in the osmolarity of the Ringer's solution following reductions in the sodium chloride concentrations were countered by substitutions with equimolar concentrations of choline chloride.

TABLE 10.

Effect of etorphine on the electrical properties of the frog's sartorius muscle.

Etorphine concentration (M)	Time in Etorphine (Min)	Resting Potential (mV)	Action Potential			
			Amplitude (mV)	Maximum Rate of Rise (V/sec)	Maximum Rate of Fall (V/sec)	Duration at -40 mV (ms)
10^{-5}	0	91.7	129.5	483.0	165.6	0.85
	5	90.5	129.8	556.9	166.9	0.78
	10	90.3	123.1	509.2	158.4	0.79
	20	91.4	126.4	512.3	166.9	0.78
	30	85.7	117.1	441.1	149.7	0.83
10^{-4}	0	85.5	118.9	387.1	132.3	1.03
	5	88.8	117.0	386.9	118.5	0.93
	10	83.3	88.7	237.2	79.4	1.01
	20	89.9	109.1	336.0	99.7	0.99
	30	89.7	108.2	306.1	92.3	1.07
10^{-3}	0	79.1	112.2	433.8	148.2	0.88
	5	81.8	93.1	251.5	77.3	1.14
	10	85.4	98.8	273.1	80.0	1.05
	20	83.6	87.1	191.5	46.9	1.40
	30	85.2	96.1	232.1	58.0	1.39

Note: Means ± S.E.M. (n=1).

4.3.2.1 Tension Studies:

The Na^+ concentration in the Ringer's solution could be lowered from a normal value of 111.8 mM down to about 44.7 mM (a 60% reduction) without appreciably reducing the twitch size; in fact, under these conditions a slight twitch potentiation accompanied by a small contracture was instead often observed (Fig. 27). However, when the extracellular Na^+ ion concentration was lowered to 33.5 mM or less (i.e., >70% reduction) it often caused reductions in the 'control' twitch height that ranged from a slight to a complete inhibition of the response (Fig. 27).

Fig. 28 shows that the peak twitch potentiation produced by methadone (5 x 10^{-5} M) is reduced progressively with progressive reductions in extracellular Na^+ concentrations. The twitch amplitude, in presence of the opioid and very low extracellular levels of Na^+ (<44.7 mM), showed a rapid decline in magnitude until the response was blocked. This effect was seen even with concentrations of the opioid that produced very little twitch potentiations, e.g., 2 x 10^{-5} M (Fig. 29). Another interesting feature of this phenomenon was an instantaneous reversal of the depressant effect and manifestation of the potentiated twitch response when the low-Na^+ Ringer's solution containing the opioid was replaced

5 min

500 mg

$[Na]_O = 100.6$ 89.4   78.3   67.1   55.9   44.7   33.5   22.4
(mM)
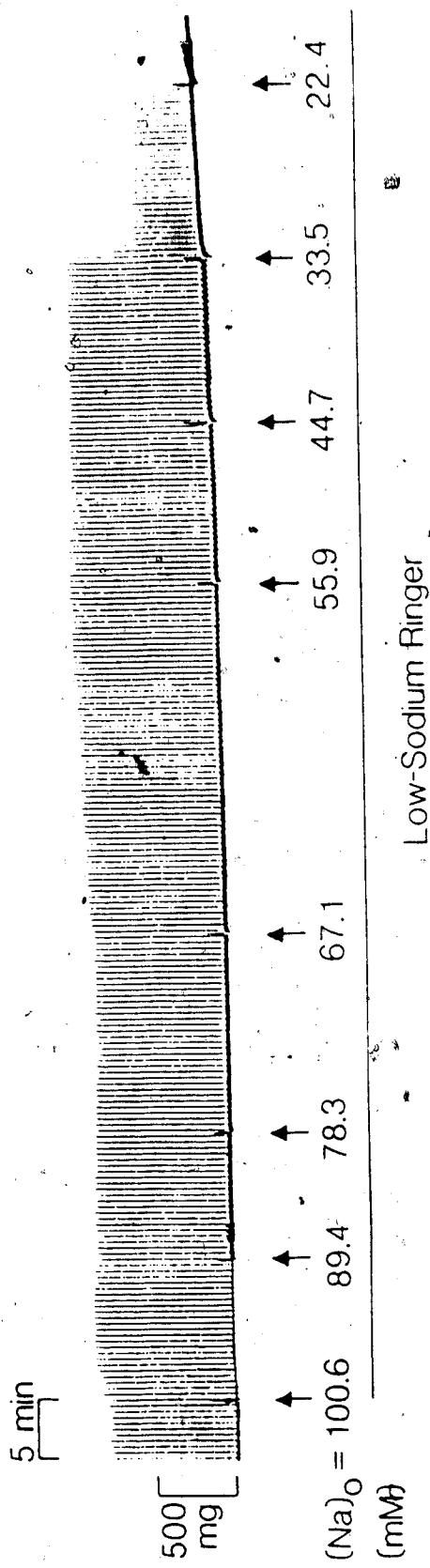
Low-Sodium Ringer

Figure 27.   Effect of low-sodium Ringer's solution on the twitch of the frog's toe muscle.   The twitch responses were elicited by single electrical pulses once every 30 sec.   The above illustration was obtained from one experiment of a series of 3 repetitions (i.e.   N = 3).

5 min

500 mg

Methadone $(5 \times 10^{-5}$ M) in NR
$(Na)_o = 111.8$ mM

w.o.

Methadone $(5 \times 10^{-5}$ M) in LSR
$(Na)_o = 78.3$ mM

w.o.

Methadone $(5 \times 10^{-5}$ M) in LSR
$(Na)_o = 55.9$ mM

w.o.

Methadone $(5 \times 10^{-5}$ M) in LSR
$(Na)_o = 47.5$ mM

w.o.

Methadone $(5 \times 10^{-5}$ M) in LSR
$(Na)_o = 44.7$ mM

w.o.
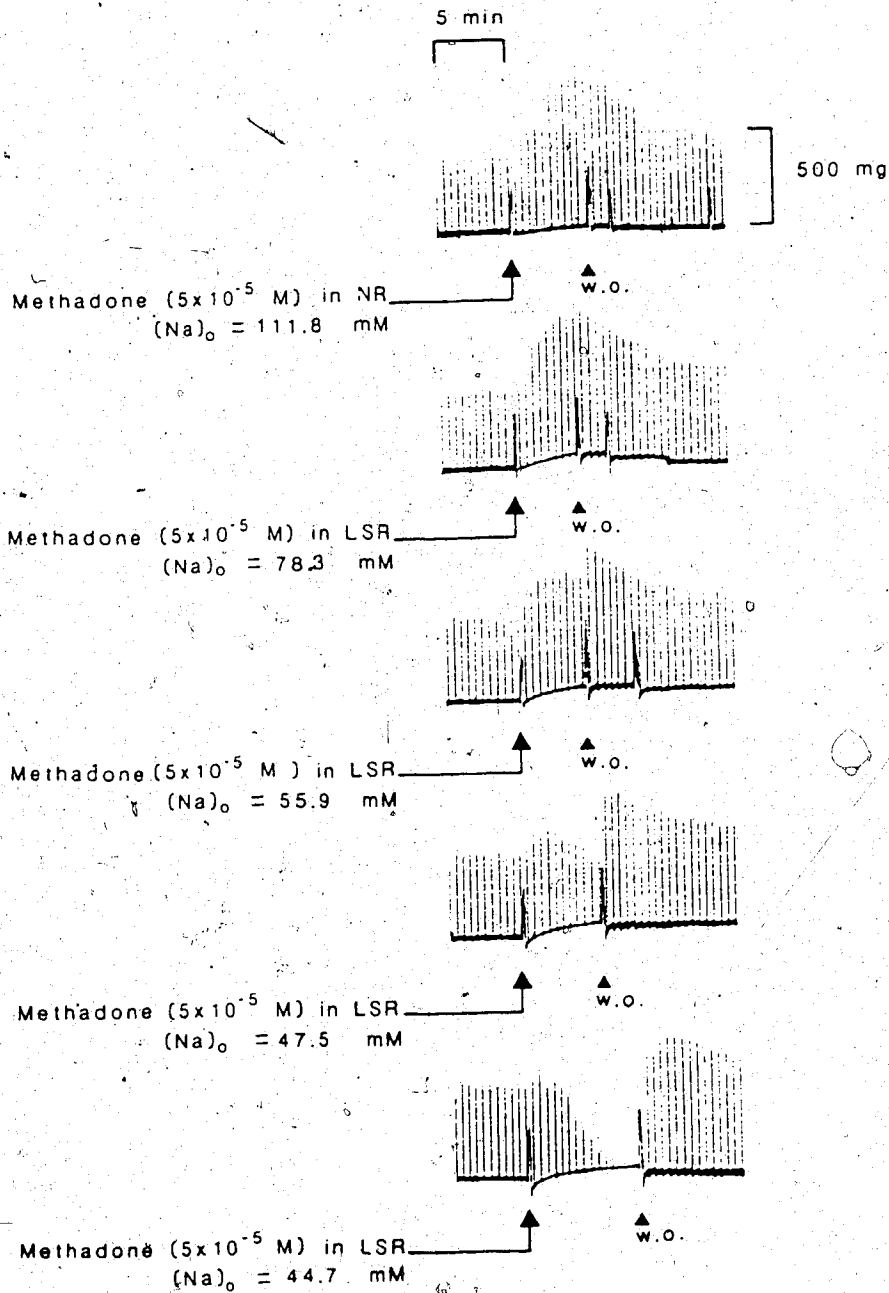
Figure 28. Progressive decrease in the peak twitch potentiation by methadone (5×10^{-5} M) in the frog's toe muscle, with a progressive reduction in the extracellular Na^+ concentration. The twitch responses were elicited by single electrical pulses once every 30 sec. The above illustration was obtained from one experiment of a series of 4 repetitions (i.e., N = 4).
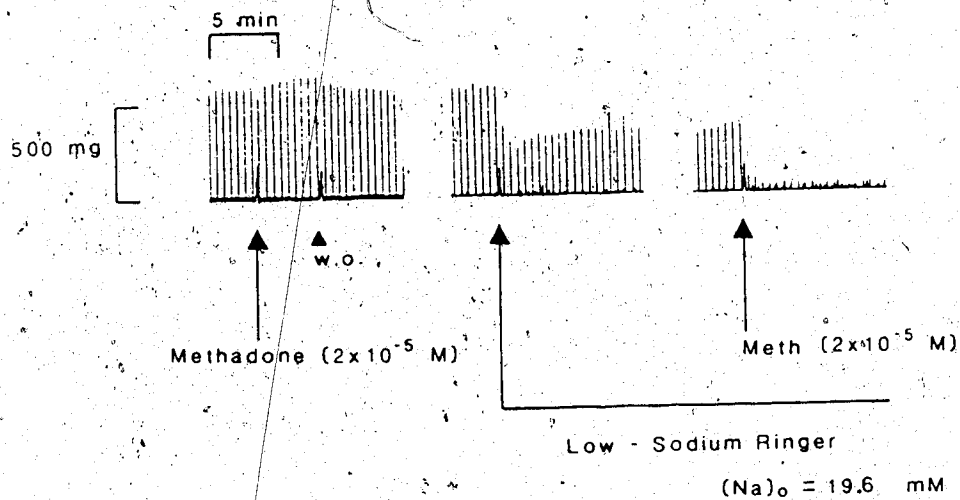
Figure 29. Depressant effect of a low concentration of methadone (2 x 10^{-5} M) on the twitch of the frog's toe muscle in presence of low-Na$^+$ Ringer's solution ([Na]$_o$ = 19.6 mM). The twitch responses were elicited by single electrical pulses once every 30 sec. The above illustration was obtained from one experiment of a series of 3 repetitions (N = 3).

with normal Ringer's solution (Fig. 28). This effect could be observed even in the continued presence of methadone provided that the normal concentration of Na^+ (viz., 111.80 mM) was made available to the muscle (Fig. 30).

Similar effects were also observed with morphine (10^{-4} M) and meperidine (10^{-4} M), when tested separately (N = 2, each).

4.3.2.2 Electrophysiological Studies:

The rapid twitch depression produced by opioids when tested on frog toe muscles bathed in low-Na^+ Ringer's solution prompted us to study their effects on the electrical properties of the muscle fibre membrane under similar experimental conditions. For this purpose, extracellular stimulation and intracellular recording techniques were used. These experiments were performed with the frog sartorius muscle.

Methadone (5 x 10^{-5} M), when tested on muscles bathed in regular Ringer's solution produced changes that were essentially similar to those produced by other opioids, viz., a decrease in the action potential amplitude, decreases in the maximal rate of rise and fall of the action potential and an increase in the action potential duration (Fig. 31). These changes were reversed following washout of the drug from the
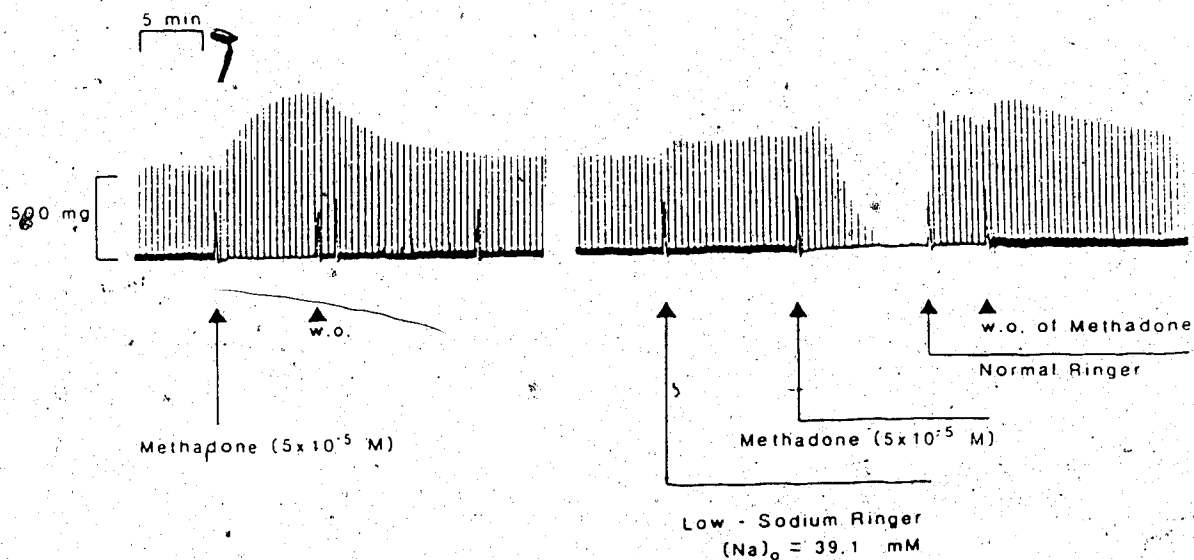
Figure 30. Depressant effect of methadone (5 x 10^{-5} M), on the twitch of the frog's toe muscle, in the presence of low-Na$^+$ Ringer's solution [Na]$_0$ = 39.1 mM; and its instantaneous reversal on exposure to normal Ringer's solution in the continued presence of the opioid. The twitch responses were elicited by single electrical pulses once every 30 sec. The above illustration was obtained from one experiment of a set of 2 repetitions (i.e., N = 2).
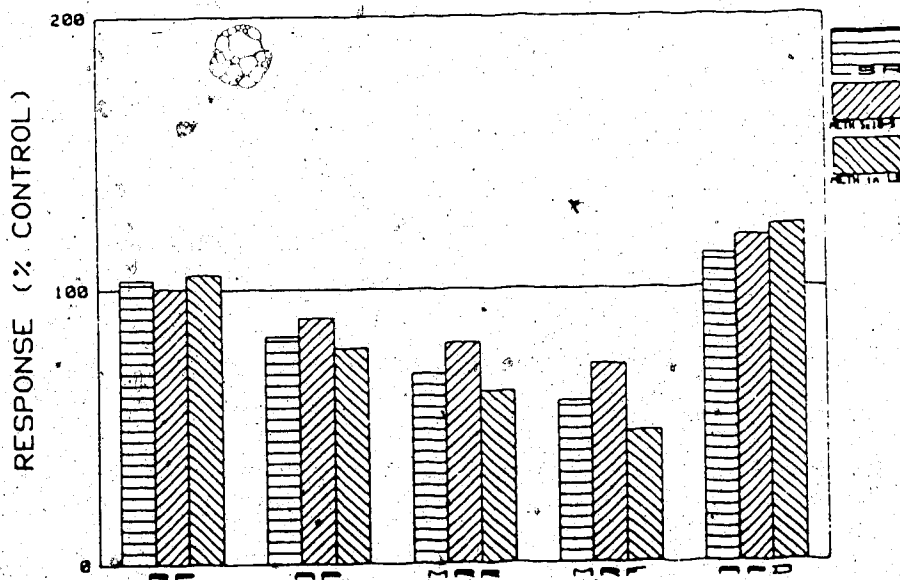
Figure 31. Effects of methadone (5×10^{-5} M) and low-Na$^+$
Ringer's solution ($[Na]_o$ = 44.7 mM), tested
individually or in combination, on the electrical
properties of the frog's sartorius muscle fibre
membrane. Electrical activity recorded at 5 min
following exposure to the opioid, low-Na$^+$ Ringer's
solution (LSR) or to a combination of the two. Results
obtained in each experiment were calculated separately,
means ± S.E.M. calculated and used to produce the graph
shown. RP, resting membrane potential; AP, action
potential amplitude; MRR, maximum rate of rise of the
action potential; MRF, maximum rate of fall of the
action potential; and APD, action potential duration
measured at -40 mV. Number of muscles (N) = 3.

muscle chamber. Low-Na$^+$ Ringer's solution ([Na]$_0$ = 44.7 mM) produced changes in the electrical parameters of the muscle fibre membrane that were qualitatively similar to those produced by the opioid (Fig. 31). When a combination of the two, viz., methadone dissolved in low-Na$^+$ Ringer's solution, was tested on the muscle, the effects appeared to be greater than either alone (Fig. 31). Thus, methadone in low-Na$^+$ Ringer's solution causes a marked depression of all the measured parameters of the action potential excepting for the action potential duration which is enhanced (Fig. 31, 32).

4.3.3 Interaction between Opioids and Ca^{++}:

4.3.3.1 Effect of methadone on K$^+$-induced contractures.

Contractures evoked by high-K$^+$ Ringer's solution ([K]$_0$ = 25 mM) were partially inhibited by methadone (8 x 10^{-5} M), although the twitch responses evoked in the same muscles were augmented by the opioid (Fig. 33). In a couple of instances when the high-K$^+$ solution was added to the muscle following incubation of the latter with the opioid for 20 min, it was observed that the contracture was reduced to about 50% of its original height. Repeated applications of the high-K$^+$ solution (once every 20 min for about 4 hrs) to the muscle in

300 V/sec

50 mV

5 msec
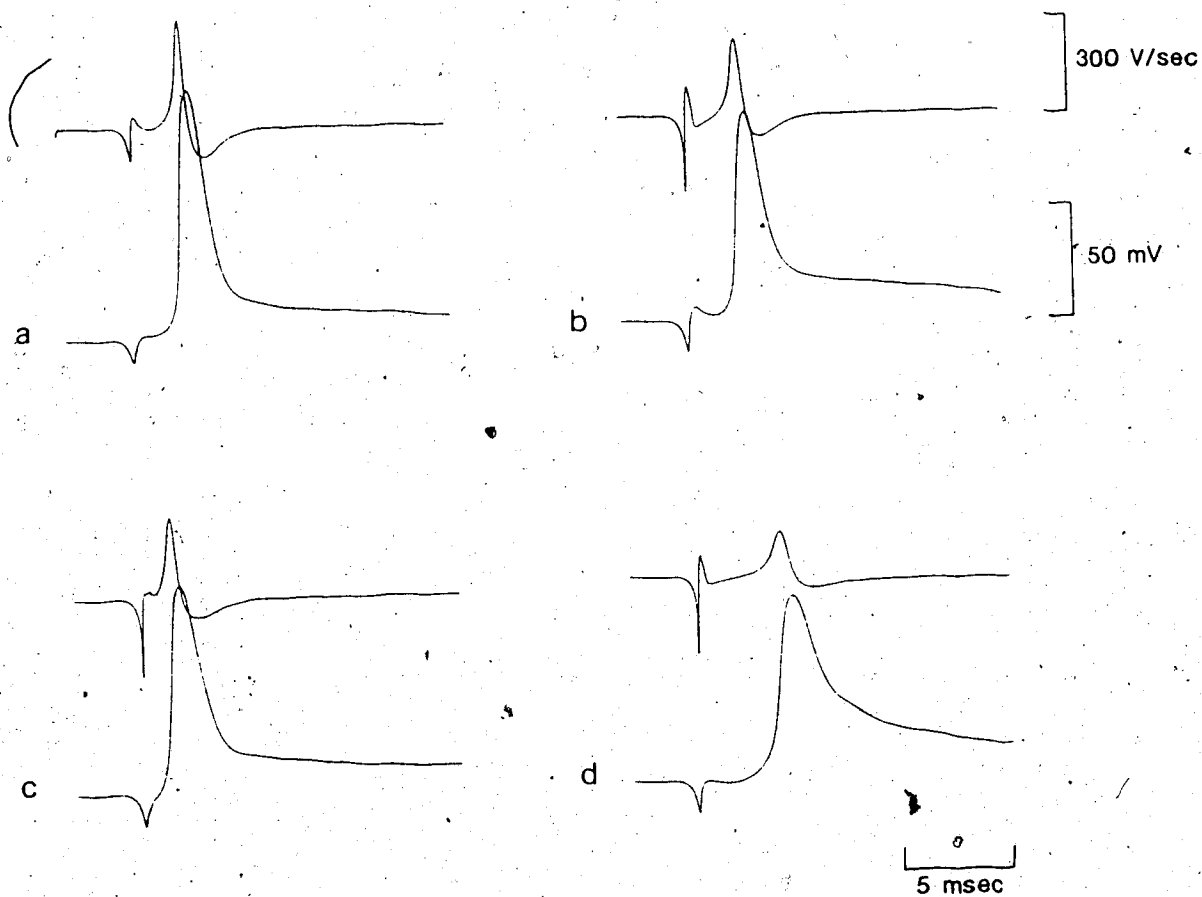
Figure 32. Effects of methadone, low-Na$^+$ Ringer's solution, or a combination of the two on the intracellularly recorded action potentials of frog's sartorius muscle strips. Upper traces, 1st differential, dV/dt; lower traces, action potential. a, control responses recorded in normal Ringer's solution; b, methadone (5 x 10^{-5} M) - effects obtained in normal Ringer's solution within 5 min of the opioid's exposure to the muscle; c, responses to low-Na$^+$ Ringer's solution, [Na]$_0$ = 44.7 mM; and d, responses to methadone dissolved in the low-Na$^+$ Ringer's solution, and obtained within 5 min of the opioid's exposure to the muscle.
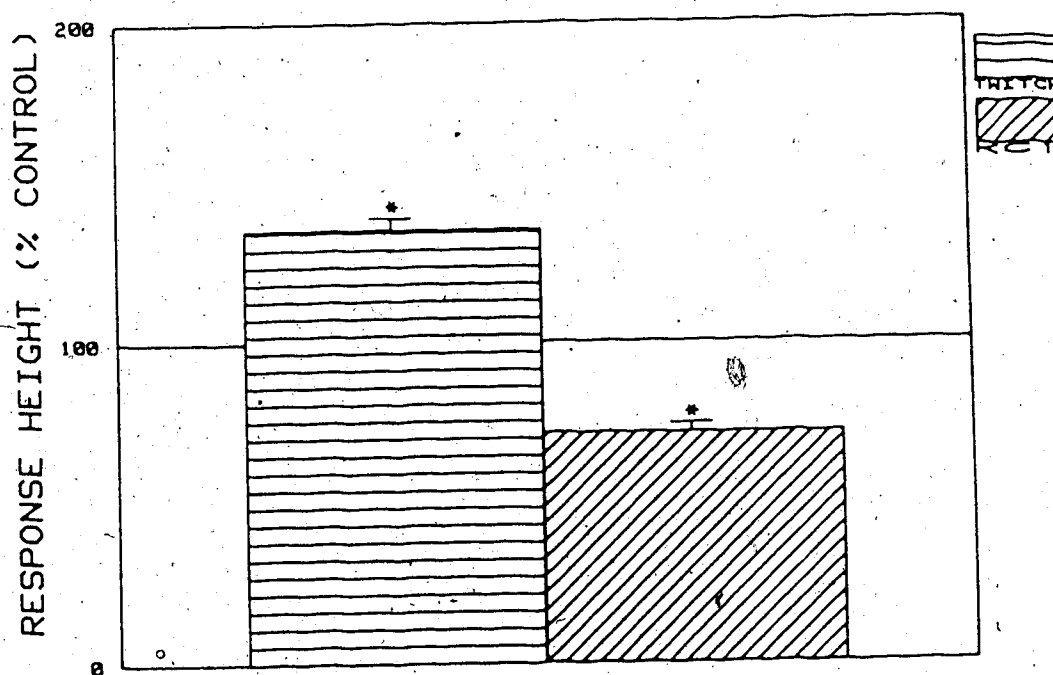
Figure 33. Inhibition of K^+(25 mM)-induced contractures by a twitch-potentiating concentration of methadone (viz., 8×10^{-5} M) in the frog's toe muscle. Contractures and twitches evoked respectively by high-K^+ Ringer's solution and electrical stimulation (once every 30 sec) were recorded using the same muscles. Means ± S.E.M., N = 8. * indicates a significant difference from control at p=0.05. Paired t-tests were conducted.

the continued presence of methadone did not cause any additional reductions in the heights of the contractures (N = 2).

4.3.3.2 Effect of D-600 on twitch potentiation by opioids.

D-600, a slow Ca^{++} channel blocking agent, was tested for effects on the opioid-induced twitch potentiations in the frog's toe muscle. The antagonist was employed in two concentrations, viz., 10^{-4} or 3 x 10^{-4} M. The twitch potentiations produced by various opioids, viz., methadone (5 x 10^{-5} M), meperidine (4 x 10^{-4} M), or morphine (10^{-4} M) were not affected by prior incubation of the muscle for 30 min with either concentrations of D-600 (Table 11).

In two experiments, toe muscles were incubated with D-600 (3 x 10^{-4} M) and were repeatedly exposed, once every 15-20 min, for periods of 30 sec each time to a high-K^+ Ringer's solution. The K^+-contractures thus elicited in the continued presence of D-600 progressively decreased in magnitude until it was down to 20-30% of the control response. At this time the twitch responses of the muscle were, however, unaltered, and methadone (5 x 10^{-5} M) continued to exhibit its usual twitch potentiating ability.

TABLE 11.

Effect of D-600 on the twitch potentiation produced by opioids in the isolated frog toe muscle.

Opioid, Concentration (M)		n	% Twitch Height (control = 100%)			
			D-600		Opioid	Opioid + D-600
			10^{-5} M	3×10^{-5} M		
Methadone,	5×10^{-5}	2	90.7 ± 5.3	---	119.1 ± 3.7	110.7 ± 2.6
Methadone,	5×10^{-5}	6	---	98.4 ± 2.2	140.9 ± 5.8	133.5 ± 3.0
Meperidine,	4×10^{-5}	4	95.5 ± 5.0	---	145.0 ± 15.7	145.9 ± 14.1
Morphine,	1×10^{-5}	4	---	100.0 ± 0.0	126.4 ± 4.4	136.4 ± 5.5

Note : The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M.
The calculated t-value was not significant ($P < 0.05$).

4.3.3.3 Effect of high-Ca^{++} Ringer's solution on opioid-induced twitch potentiations.

Increasing the Ca^{++} concentration of the Ringer's solution bathing the muscle from 1.08 mM to 4.32 mM or above caused an initial decrease in twitch tension which often recovered to 'control' values within 30 - 40 min of incubation. Therefore, toe muscles were exposed to the high-Ca^{++} Ringer's solution ($[Ca]_o$ = 2.16 - 10.08 mM) for 40 min before testing the effects of opioids (in the continued presence of the high-Ca^{++} solution) on the twitch.

The twitch potentiation produced by methadone (10^{-5} - 3 x 10^{-4} M) was essentially unaltered by increasing Ca^{++} concentration in the bath up to 6.48 mM, but further increases in the extracellular Ca^{++} concentration caused an almost complete inhibition of the effect of methadone (Table 12).

Single experiments were performed using other opioids, viz., morphine (10^{-4} M) or meperidine (10^{-4} M), and the same extracellular concentration of Ca^{++} (i.e., 8.64 mM). It was again observed that the twitch potentiating effects of the opioids are effectively countered by an increased concentration of extracellular Ca^{++}.

TABLE 12.

Effect of high extracellular calcium concentration on the twitch potentiation produced by methadone in the isolated frog toe muscle.

Calcium concentration (mM)	% Twitch Potentiation by Methadone			
	1×10^{-5} M	3×10^{-5} M	1×10^{-4} M	3×10^{-4} M
1.08 (control)	104.2 ± 4.2	114.2 ± 4.5	144.1 ± 10.2	173.3 ± 11.3
2.16	107.2 ± 2.9	115.2 ± 3.5	149.3 ± 4.9	185.5 ± 12.2
1.08 (control)	100.1 ± 1.1	110.4 ± 3.6	144.1 ± 12.6	164.1 ± 20.4
4.32	104.6 ± 1.8	121.2 ± 4.0	153.6 ± 9.8	173.8 ± 17.1
1.08 (control)	94.2 ± 6.7	111.0 ± 9.8	136.0 ± 6.3	152.0 ± 11.9
6.48	103.5 ± 2.2	116.3 ± 4.2	143.5 ± 16.8	150.3 ± 31.6
1.08 (control)	105.8 ± 1.0	113.8 ± 1.9	134.1 ± 5.9	133.2 ± 10.8
8.64	100.0 ± 0.0*	104.9 ± 2.6*	106.3 ± 3.2*	94.3 ± 6.9*
1.08 (control)	105.1 ± 2.4	120.2 ± 5.7	156.7 ± 14.1	189.1 ± 20.5
10.08	99.1 ± 1.8*	102.8 ± 5.3*	117.8 ± 10.0*	110.9 ± 12.0*

Note : The muscles were incubated with the high-calcium Ringer's (HCR) solution for 40 min before testing the effect of the opioid on the muscle in the continued presence of the HCR solution.
The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M.   (n=6).   Paired t-tests were conducted.
* Means with and without HCR solution significantly different at $p < 0.05$.

4.3.3.4 Effect of high-Ca^{++} Ringer's solution on the methadone-induced changes in the electrical properties of the muscle fibre membrane:

Intracellular recording experiments were conducted to see if a high - Ca^{++} Ringer's solution (HCR) could antagonize methadone - effects on the electrical properties of the membrane as it antagonized the opioid - effects on the twitch (Table 12).

In this set of experiments, the electrical responses of the muscle, viz., the resting membrane potential, the action potential amplitude, the maximum rates of rise and fall of the action potential, and the action potential duration (measured at -40mV) were recorded before and after methadone (3.2×10^{-4} M) addition. The electrical events were recorded at 5, 10, 20, and 30 min following the addition of the drug. Using fresh sartorii muscles, the electrical parameters of the muscle fibre membrane were recorded first in normal Ringer's solution (NR) and then in HCR solution; the responses being recorded after equilibration of the muscle in the two solutions for 60 min and 40 min, respectively. The same muscles were next exposed to methadone (3.2×10^{-4}M) dissolved in HCR ([Ca]$_0$ = 8.64 mM or 10.08 mM) and the electrical events were recorded periodically as described above . No attempt was made to record both the 'control' responses and the 'test'

responses to methadone from the same muscles because the muscles never recovered completely from the 'control' methadone effects even when washed with fresh regular Ringer's solution once every 10-15 min for over 2 hrs.

Methadone $(3.2 \times 10^{-4}M)$-effects on the electrical parameters of the muscle fibre membrane were similar to the effects produced by other opioids (Ch. 4.3.1). Methadone did not affect the resting membrane potential up to 30 min following its exposure to the muscle (Fig. 34). It produced a slight but significant reduction of the action potential amplitude at the end of a 20 min exposure period (Fig. 35). The maximum rates of rise and fall of the action potential were depressed within 5 min of exposure to the muscle (Fig. 36, 37), and simultaneously there was a significant prolongation of the action potential duration (Fig. 38).

Elevation of extracellular Ca^{++} levels to 8.64 mM by itself did not significantly affect any of the measured electrical properties of the muscle fibre membrane. HCR ($[Ca]_o = X8$) also did not antagonize any of the changes in the electrical properties of the muscle fibre membrane produced by methadone $(3.2 \times 10^{-4}M)$ within 5 min of its exposure to the muscle (Figs. 34 - 38).
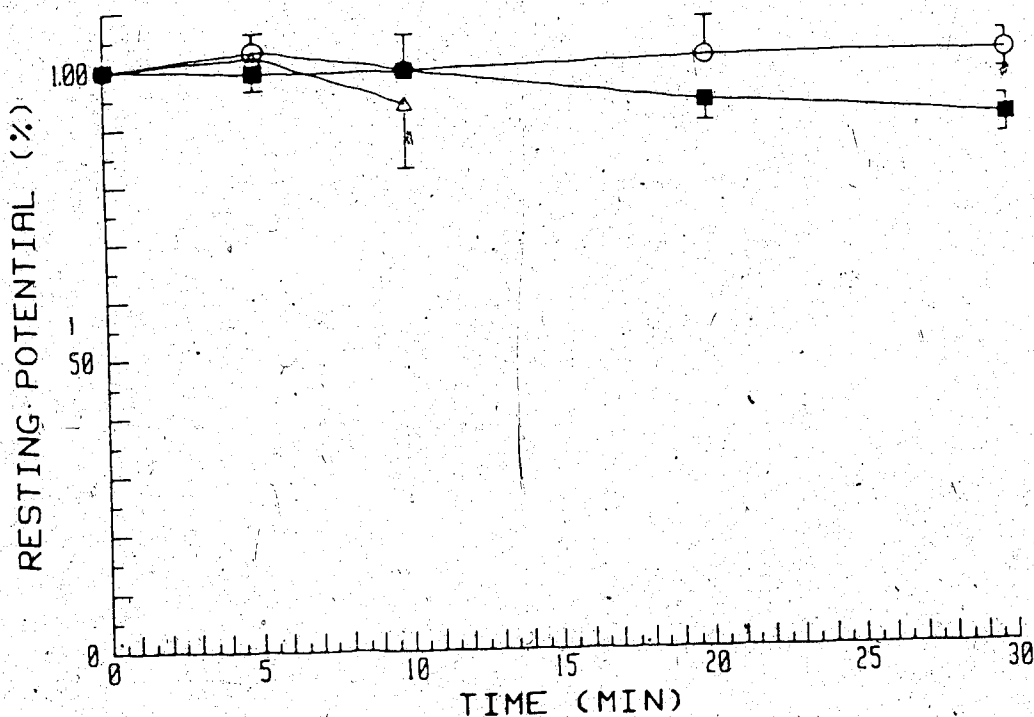
Figure 34. Lack of effect of methadone (3.2 x 10⁻⁴ M) on the resting membrane potential of frog's sartorius muscle strips. ■, methadone in normal Ringer's (NR) solution ($[Ca]_o$ = 1.08 mM), N = 7 muscles; O, methadone in high-Ca⁺⁺ ($[Ca]_o$ = 8.64 mM) Ringer's (HCR) solution, N = 3 muscles; Δ, methadone in HCR ($[Ca]_o$ = 10.08 mM) solution, N = 3 muscles. All muscles exposed to methadone (in NR or HCR) at time 0. Results obtained in each experiment were calculated separately, means ± S.E.M. calculated and used to produce the graph shown.
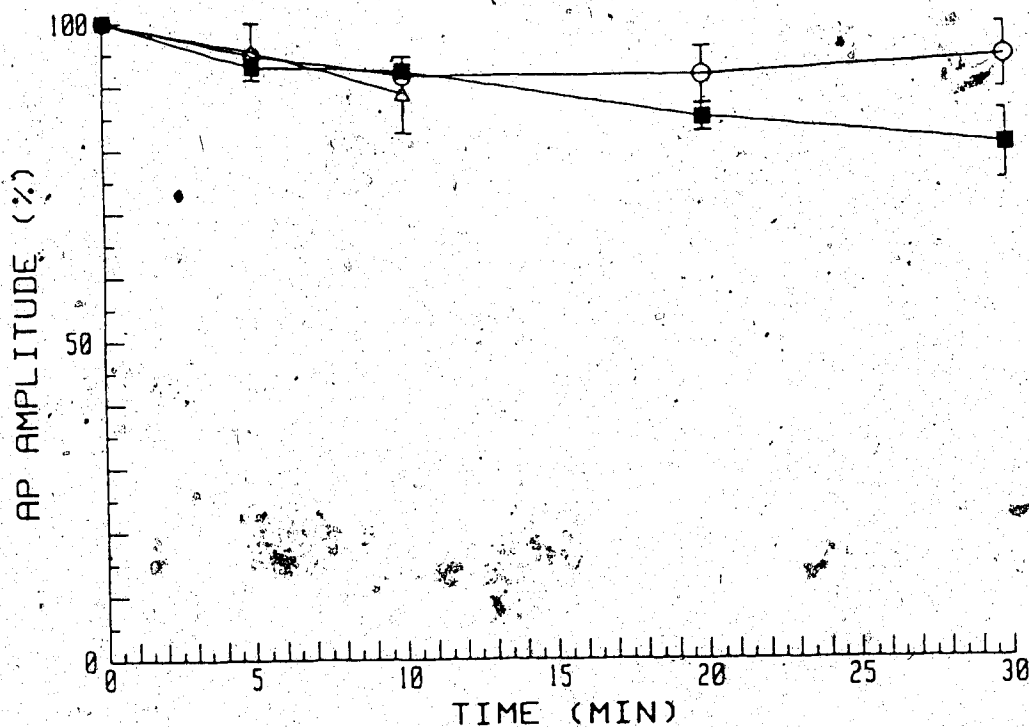
Figure 35. Lack of an antagonistic effect of high extracellular concentration of Ca^{++} on the methadone (3.2×10^{-4} M)-induced changes in the action potential amplitude of frog's sartorius muscle strips. ■, methadone in normal Ringer's (NR) solution ($[Ca]_0 = 1.08$ mM), N = 7 muscles; O, methadone in high-Ca^{++} Ringer's (HCR) solution ($[Ca]_0 = 8.64$ mM), N = 3 muscles; Δ, methadone in (HCR) solution ($[Ca]_0 = 10.08$ mM), N = 3 muscles. All test muscles were incubated in HCR solution for 40 min before testing the opioid. Results obtained in each experiment were calculated separately, means ± S.E.M. calculated and used to produce the graph shown. An unpaired t-test (one tail) was conducted.
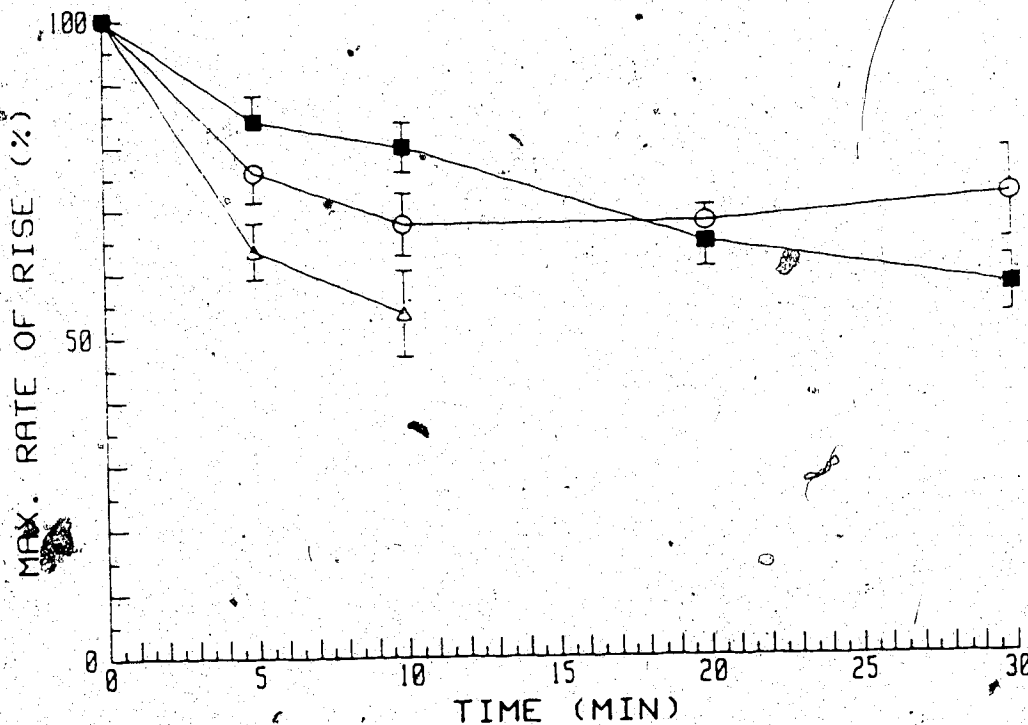
Figure 36. Lack of an antagonistic effect of high extracellular concentration of Ca⁺⁺ on the changes produced by methadone (3.2 x 10⁻⁴ M) in the maximum rate of rise of the action potential amplitude of frog's sartorius muscle strips. ■, methadone in normal Ringer's (NR) solution ($[Ca]_o$ = 1.08 mM), N = 7 muscles; O, methadone in high-Ca⁺⁺ Ringer's (HCR) solution ($[Ca]_o$ = 8.64 mM), N = 3 muscles; Δ, methadone in (HCR) solution ($[Ca]_o$ = 10.08 mM), N = 3 muscles. All test muscles were incubated in HCR solution for 40 min before testing the opioid. Results obtained in each experiment were calculated separately, means ± S.E.M. calculated and used to produce the graph shown. An unpaired t-test (one tail) was conducted.

Figure 37. Lack of an antagonistic effect of high extracellular concentration of Ca^{++} on the changes produced by methadone (3.2×10^{-4} M) in the maximum rate of fall of the action potential amplitude of frog's sartorius muscle strips. ■, methadone in normal Ringer's (NR) solution ($[Ca]_o = 1.08$ mM), N = 7 muscles; O, methadone in high-Ca^{++} Ringer's (HCR) solution ($[Ca]_o = 8.64$ mM), N = 3 muscles; Δ, methadone in (HCR) solution ($[Ca]_o = 10.08$ mM), N = 3 muscles. All 'test' muscles were incubated in HCR solution for 40 min before testing the opioid. Results obtained in each experiment were calculated separately, means ± S.E.M. calculated and used to produce the graph shown. An unpaired t-test (one tail) was conducted.

Figure 38. Effect of high extracellular concentration of Ca^{++} on the methadone-induced changes in the action potential duration (measured at -40 mV) of frog's sartorius muscle strips. ■, methadone in normal Ringer's (NR) solution ($[Ca]_0$ = 1.08 mM), N = 7 muscles; O, methadone in high-Ca^{++} Ringer's (HCR) solution ($[Ca]_0$ = 8.64 mM), N = 3 muscles; Δ, methadone in (HCR) solution ($[Ca]_0$ = 10.08 mM), N = 3 muscles. All test muscles were incubated in HCR solution for 40 min before testing the opioid. Results obtained in each experiment were calculated separately, means ± S.E.M. calculated and used to produce the graph shown. * indicates a significant difference from control methadone response at p = 0.05. An unpaired t-test (one tail) was conducted.

HCR ($[Ca]_o$ = 10.08 mM) also did not affect the resting membrane potential or the action potential amplitude but it did depress significantly the maximum rates of rise and fall of the action potential and caused a significant increase in the duration of the action potential (Figs. 34 - 38). However even this higher concentration of extracellular Ca did not antagonize any of the changes produced in the electrical parameters of the muscle by within 5 min of its expos the muscle. Under these experimental condition muscles become inexcitable after about 10 min.

Thus, elevation of extracellular Ca^{++} levels to 8.64 or 10.08 mM, antagonizes the twitch potentiation by methadone, but does not antagonize any of the changes produced in the electrical parameters of the muscle by the opioid within 5 min of its exposure to the muscle.

4.3.3.5 Effect of low-Ca^{++} Ringer's solution on opioid-induced twitch potentiations.

A 70% reduction in the extracellular Ca^{++} concentration (from 1.08 mM to 0.324 mM) caused no observable effects on the twitch of the frog's toe muscle. Further decreases in the concentration of the above ion, however, produced enhanced twitches that

were at times superimposed on small contractures.

The effect of reduced extracellular concentration of Ca^{++} on the twitch potentiation produced by methadone was studied. The muscle was soaked in the low-Ca^{++} solution for 20 min before testing the opioid. It was observed that reduction of the Ca^{++} concentration in the bathing solution from 1.08 mM to 0.54 mM (a 50% reduction) or to 0.216 mM (an 80% reduction) caused no change in the twitch potentiations produced by 3.2 x 10^{-4} M or 10^{-4} M methadone, respectively (Table 13).

4.3.3.6 Effect of caffeine on twitch potentiation by opioids:

Low concentrations of caffeine, viz., 4 x 10^{-4} M or 8 x 10^{-4} M did not affect the twitch potentiation produced by 5 x 10^{-5} M or 10^{-4} M methadone. Higher concentrations of caffeine (3 x 10^{-3} M) however, produced a marked attenuation of the effects of the opioids tested, viz., meperidine (10^{-4} M) or morphine (10^{-4} M). The antagonism by caffeine was reversible within an hour of its removal from the bath (Fig. 39, 40).

4.3.3.7 Effect of A23187 on twitch potentiation by opioids.

TABLE 13.

Effect of low-calcium Ringer's (LCR) solution on the twitch potentiation produced by methadone in the isolated frog toe muscle.

Methadone Concentration (M)	% Twitch Height (control = 100%)			
	LCR Solution		Methadone (in normal Ringer's)	Methadone in LCR
	0.54 (mM)	0.216 (mM)		
3.2×10^{-4}	100.0 ± 0.0	--	154.1 ± 5.9	154.5 ± 3.6
1.0×10^{-4}	--	109.4 ± 3.4	148.3 ± 9.9	148.9 ± 6.3

Note : The muscle was soaked in the LCR solution for 30 min before testing the opioid.
Means ± S.E.M. (n=4).
The calculated t-value was not significant ($p < 0.05$).
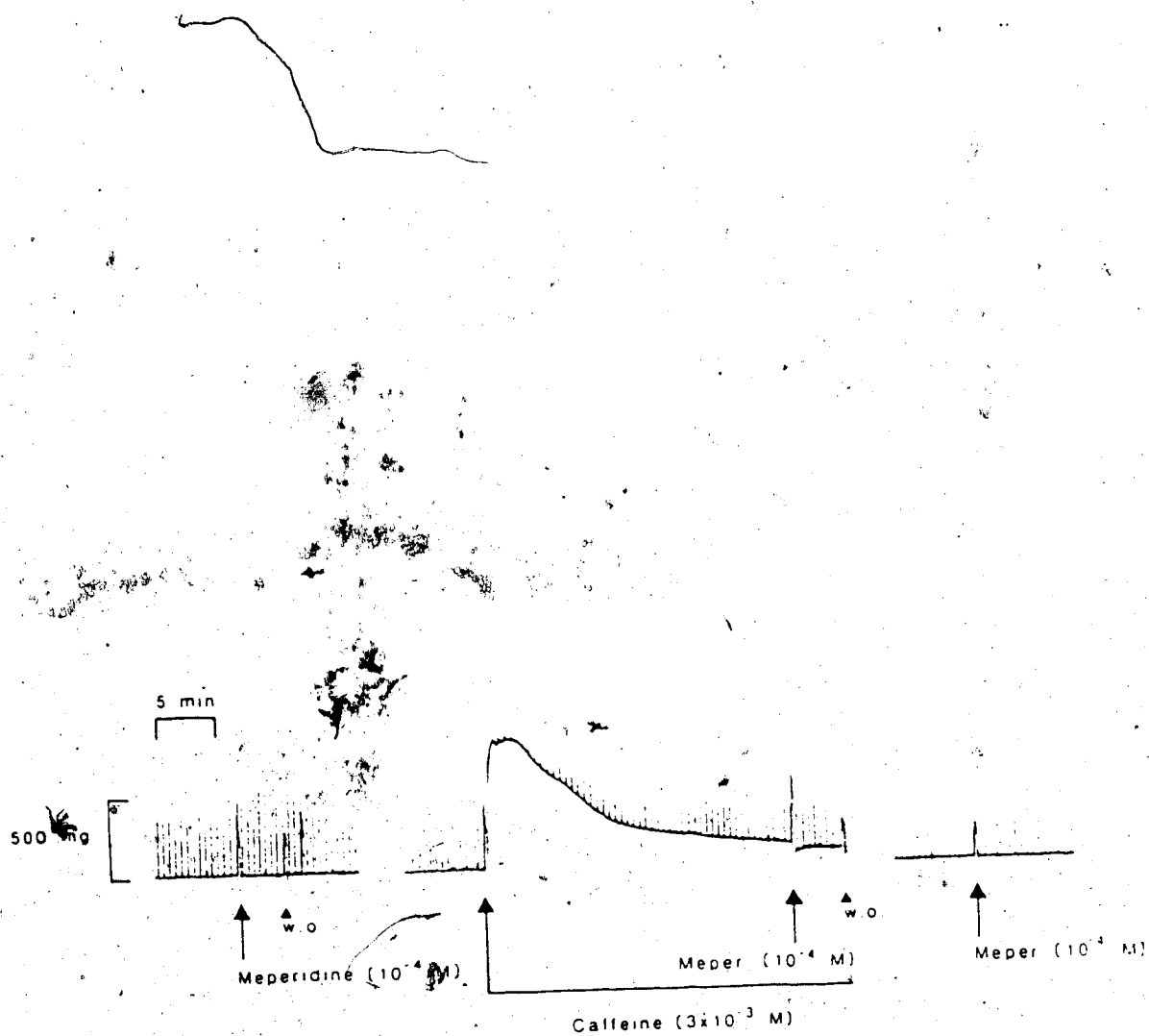
Figure 39. Antagonistic effect of caffeine (3 x 10^{-3} M) on the twitch-potentiation produced by meperidine (10^{-4} M) in the frog's toe muscle. The twitch response was elicited by single electrical shocks applied once every 30 sec. The above illustration was obtained from one experiment of a set of 4 repetitions (i.e., N = 4).
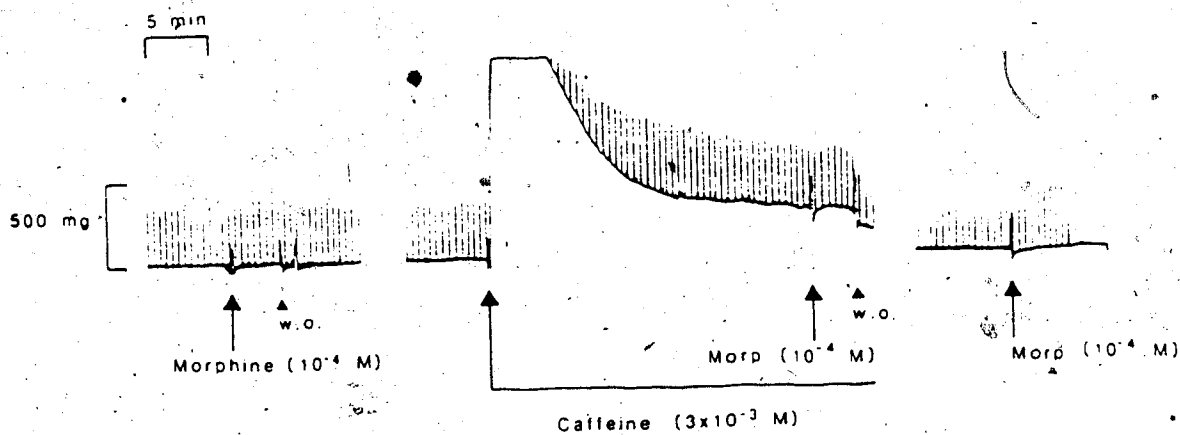
Figure 40.   antagonistic effect of caffeine (3 x 10⁻³ M) on the twitch-potentiation produced by morphine (10⁻⁴ M) in the frog's toe muscle. The twitch response was elicited by single electrical shocks applied once every 30 sec. The above illustration was obtained from one experiment of a set of 3 repetitions (i.e., N = 3).

The Ca^{++} ionophore, A-23187 (7.6 x 10^{-7} M) failed to affect the twitch potentiation produced by 5 x 10^{-5} or 10^{-4} M methadone. Higher concentrations of the ionophore, viz., 10^{-4} M or 5 x 10^{-4} M however, antagonized significantly the twitch potentiation by 10^{-4} M methadone (Table 14).

4.3.3.8 ^{45}Ca Desaturation Studies.

Effect of La^{+++}: La^{+++} was used to test the ability of our procedures to detect the displacement of Ca^{++} bound superficially to the extracellular surface of the muscle fiber membrane. Pairs of toe muscles from single frogs were incubated in Tris Ringer's solution containing ^{45}Ca for 3-4 hrs, and then efflux into a Ca^{++}-free Tris Ringer's solution was followed. One muscle from each pair acted as a drug-free control. Efflux samples from both muscles were either collected once every 5 min for 60 min in three experiments (N=3) or once every min for the first 10 min followed by once every 5 min for the next 20 min and finally once every 15 min for the next 3.5 hr in two other experiments. The efflux media were the Ca^{++}-free Tris Ringer's (CFTR) solution with or without 2 mM La^{+++}. A typical result using the latter timing is shown in Fig. 41.

La^{+++} produced an immediate increase in the rate of loss of ^{45}Ca into the efflux medium (Fig. 42). This

TABLE 14.

Effect of the calcium-ionophore, A23187, on the twitch potentiation produced by methadone (10^{-4} M) in the frogs toe muscle.

A23187 Concentration (M)	n	% Twitch Potentiation			
		Solvent	A23187	Methadone	Methadone + A23187
1×10^{-5}	4	100.0 ± 0.0	105.0 ± 5.0	216.7 ± 3.4	156.7 ± 3.4*
5×10^{-5}	3	100.0 ± 0.0	108.4 ± 20.2	246.8 ± 57.9	130.3 ± 12.7*

Note : The 'test' response to the opioid was recorded in the presence of the ionophore after the muscle had attained
a steady tone during the contracture.
The values are expressed as a percentage of the control (100%) response.
Means ± S.E.M.
* Significant difference (p < 0.05) between the mean values of the opioid-effects observed in the absence
and in the presence of the ionophore. A paired t-test was conducted.
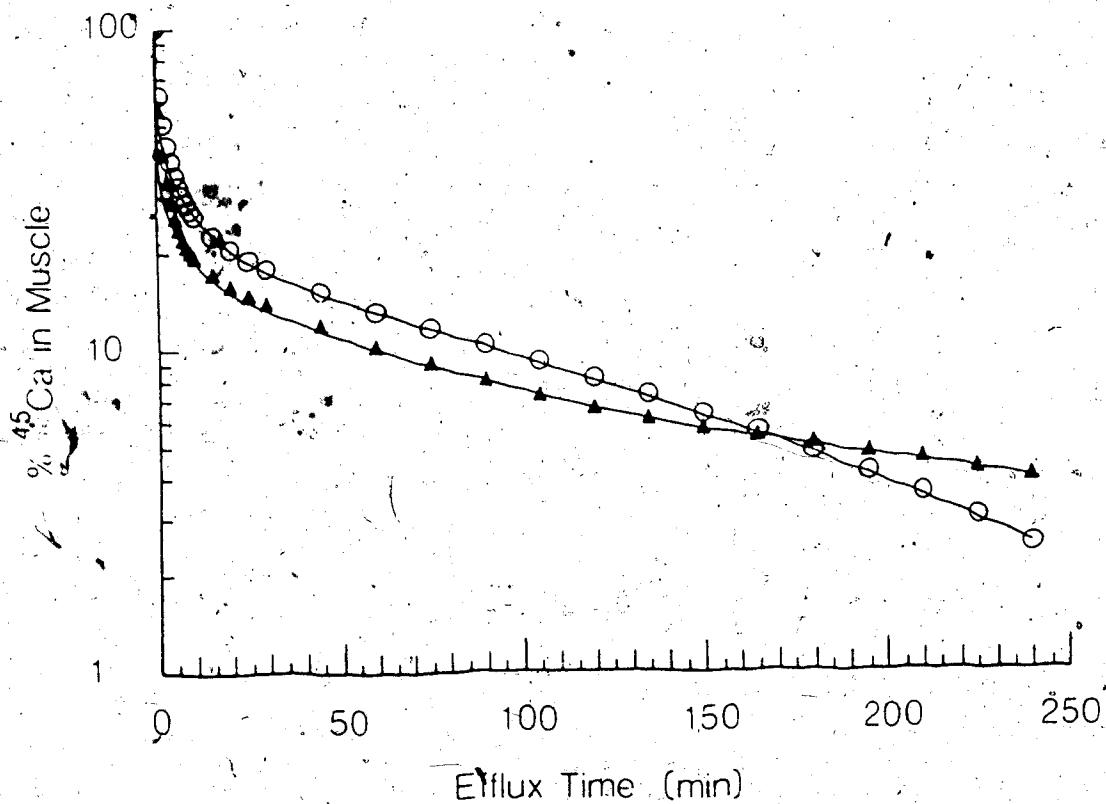
Figure 41. Effect of La^{+++} on ^{45}Ca efflux from frog's toe muscle. Results obtained with a pair of toe muscles from a single frog. The muscles were incubated in Tris Ringer's solution containing ^{45}Ca for 3 hr and then the efflux into a Ca^{++}-free Tris Ringer's solution with or without La^{+++} (2 mM) was measured. (▲), La^{+++} was present throughout the efflux period - one muscle, (O), control efflux without La^{+++}, - the other muscle.
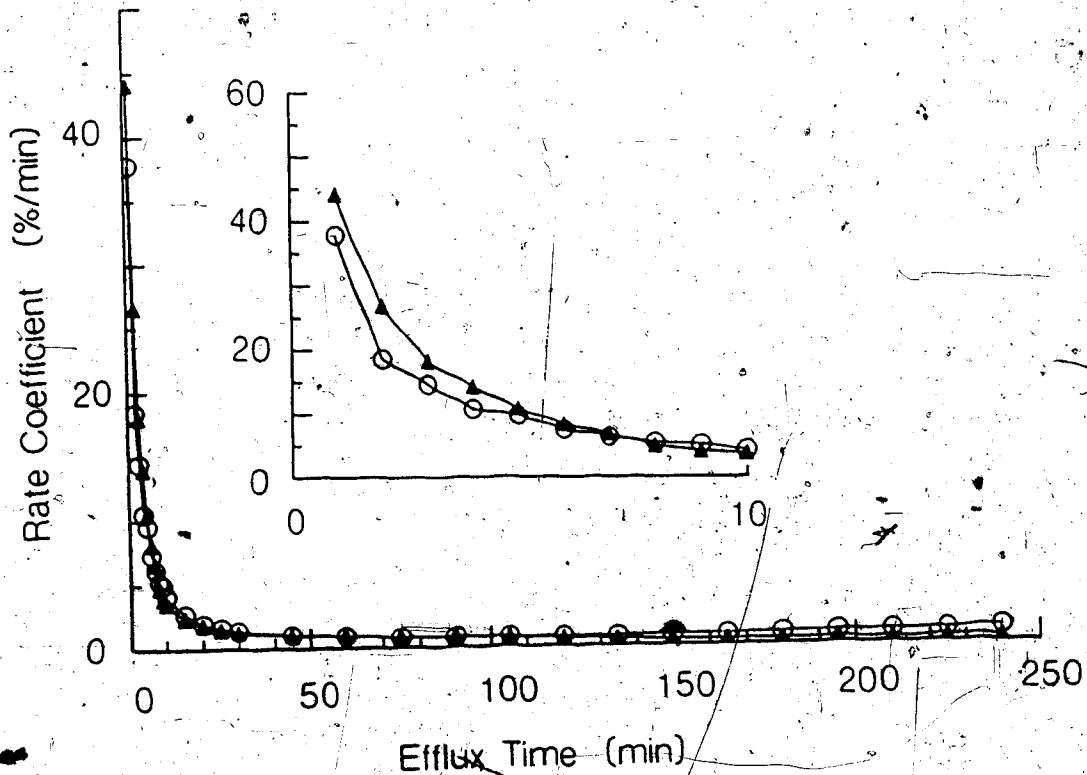
Figure 42. Efflux rate coefficient curves obtained from the data in Fig. 41. (▲, La^{+++} was present throughout the efflux period - one muscle; (O), control efflux without La^{+++} - the other muscle. Insert, time scale expansion of the first 10 min of this curve.

effect, however was not sustained; in the experiment
shown the efflux rates were equal by 5 min. This
resulted in less ^{45}Ca remaining in the muscle treated
with La^{+++} (Fig. 41). However, the ^{45}Ca efflux with
La^{+++} often decreased during the later part of the
efflux to values less than those of the control. This
occurred in the two 4 hr efflux experiments as shown in
Fig. 41 and at 20 min in one of the three 60 min efflux
experiments.

Effect of methadone (10^{-4} M): Four pairs of toe
muscles from different frogs were tested. These muscle
pairs were kept in ^{45}Ca labelled normal Ringer's
solution for 3 hr and then the efflux of ^{45}Ca into a
Ca^{++}-free normal Ringer's solution was followed.
Efflux samples from both muscles were collected once
every 5 min for 90 min. In these experiments the
efflux medium for the 'test' muscle contained methadone
(10^{-4} M) from the beginning of the efflux whereas it
was omitted from the efflux medium of the 'control'
muscle. The results obtained in these four experiments
were averaged and the average is presented in Fig. 43.

The efflux rate coefficient curves obtained using
these averages (Fig. 44) show that methadone (10^{-4} M)
caused an immediate (within 5 min) but transient
increase in the rate of loss of ^{45}Ca as compared to
drug-free controls.
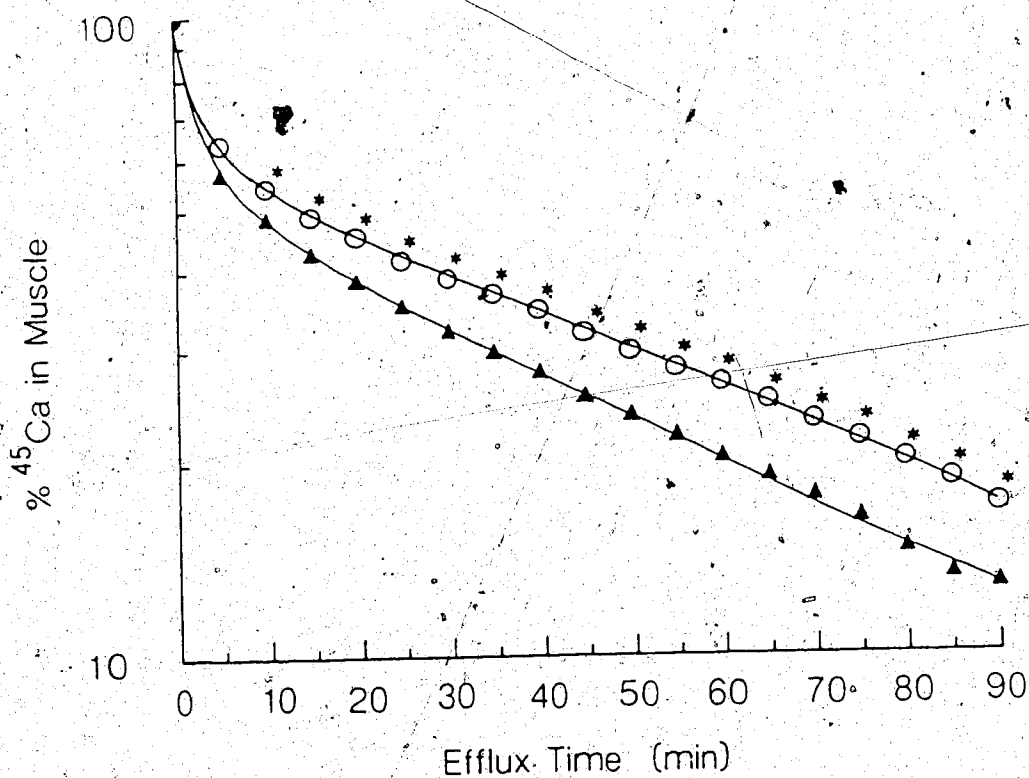
Figure 43. Effect of methadone $(10^{-4}$ M) on ^{45}Ca efflux in frog's toe muscles. ▲, methadone $(10^{-4}$ M) was present throughout the efflux period; O, control without methadone. The symbols are averages of values obtained with four toe muscle pairs (i.e., N = 4 for each curve); each muscle pair being obtained from a single animal. The differences between the paired control and test values are statistically significant (p = 0.05) at each point by an asterisk (*). A paired t-test was conducted.
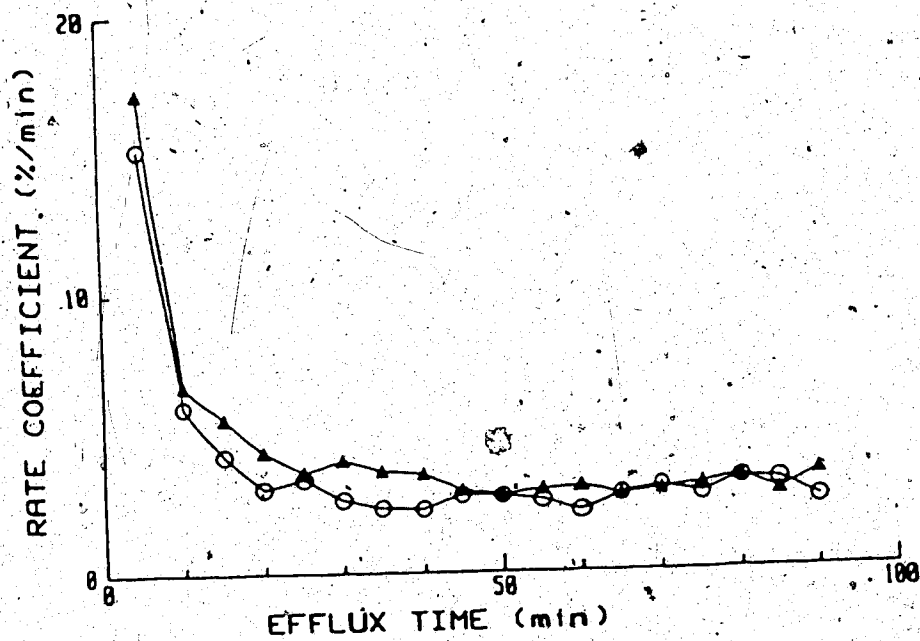
Figure 44. Efflux rate coefficient curves obtained from the data in Fig. 43. ▲, methadone (10^{-4} M) was present throughout the efflux period; O, control without methadone.

Effect of morphine (10^{-4} or 10^{-3} M): Eight pairs of toe muscles from single frogs were loaded in ^{45}Ca labelled normal Ringer's solution for 3-5 hr and the efflux of ^{45}Ca into a Ca^{++}-free normal Ringer's solution, with or without morphine (10^{-4} M, n=4 pairs or 10^{-3} M, n=4 pairs), was followed. Efflux samples were collected once every min for the first 5 min and then every 5 min for the next 55 min. Morphine caused no change in the rate of loss of the tracer into the medium during the 60 min of efflux studied. In 7 of the 8 experiments the 'control' and morphine efflux curves were identical. In the other, morphine produced a slight, transient decrease in ^{45}Ca efflux.

Effect of meperidine (10^{-4} M): The experimental procedure was similar to that described in the previous experiment with morphine except for the use of meperidine (10^{-4} M) instead of morphine. Two such experiments were conducted, and the results from 'control' and 'test' preparations were identical indicating that meperidine did not displace the superficially bound membrane Ca^{++}.

Interaction of the effects of La^{+++} (2 mM) and opioids on ^{45}Ca efflux from superficial membrane sites: The small or non-existent effects on the efflux of 'superficially bound Ca^{++} observed in the above experiments persuaded us to adopt procedures to

increase the sensitivity for detecting this displacement. To do this it seemed reasonable to enhance the concentration of ^{45}Ca bound to these superficial membrane sites. This was achieved by first reducing total Ca^{++} stores in the muscle by soaking it in nonradioactive, Ca^{++}-free Tris Ringer's solution for 3 hr; the solution being replaced once every 15 min. Soaking frog skeletal muscles in Ca^{++}-free solutions is known to reduce superficial Ca^{++} stores to low levels usually within 10 min in frog's toe muscles [250]. The muscles were next exposed to Ca^{++}-free Tris Ringer's solution to which was added 4-5 μCi/ml of ^{45}Ca. The incubation with the radioactive solution was kept relatively short, viz., 30 min, in order to minimize ^{45}Ca uptake into the deeper intracellular stores. Since the superficial sites on the membrane were initially depleted of Ca^{++} and next exposed to a radioactive solution with a very high specific activity of Ca^{++}, the amount of ^{45}Ca bound to superficial sites in these experiments was presumably much greater than in the previous experiments.

^{45}Ca efflux from both muscles of a muscle pair into a Ca^{++}-free medium was followed for only 45 min in these experiments. Efflux samples from both muscles were collected every minute for the first 10 min and then every 5 min for the next 35 min. In these

experiments the efflux medium for both muscles was drug-free and Ca^{++}-free Tris Ringer's solution (CFTR) for the first 4 min. Next, between 4 and 20 min the efflux medium for one muscle contained La^{+++} (2 mM) and that for the other muscle contained an opioid. The efflux medium for the balance of the efflux period (i.e., from 20 to 45 min) for both muscles contained both La^{+++} and the drug.

The ^{45}Ca desaturation curves showed that the superficially bound Ca^{++} was not displaced by morphine (10^{-3} M) even though there was a large reserve of Ca^{++} in this pool as indicated by its displacement with La^{+++} (Fig. 45). Two experiments were conducted with morphine and identical results were obtained. The results of one of these experiments is presented in Fig. 45.

In another two experiments the same experimental procedure was followed except for the use of meperidine (10^{-3} M) instead of morphine. The result of one of these experiments is presented in Fig. 46. The results of these experiments were the same as for morphine showing that meperidine also did not displace the superficially bound membrane Ca^{++}.

When similar experiments were carried out using methadone (10^{-4} M, n=2 pairs) a slight but significant increase in the rate of ^{45}Ca efflux from the muscle was
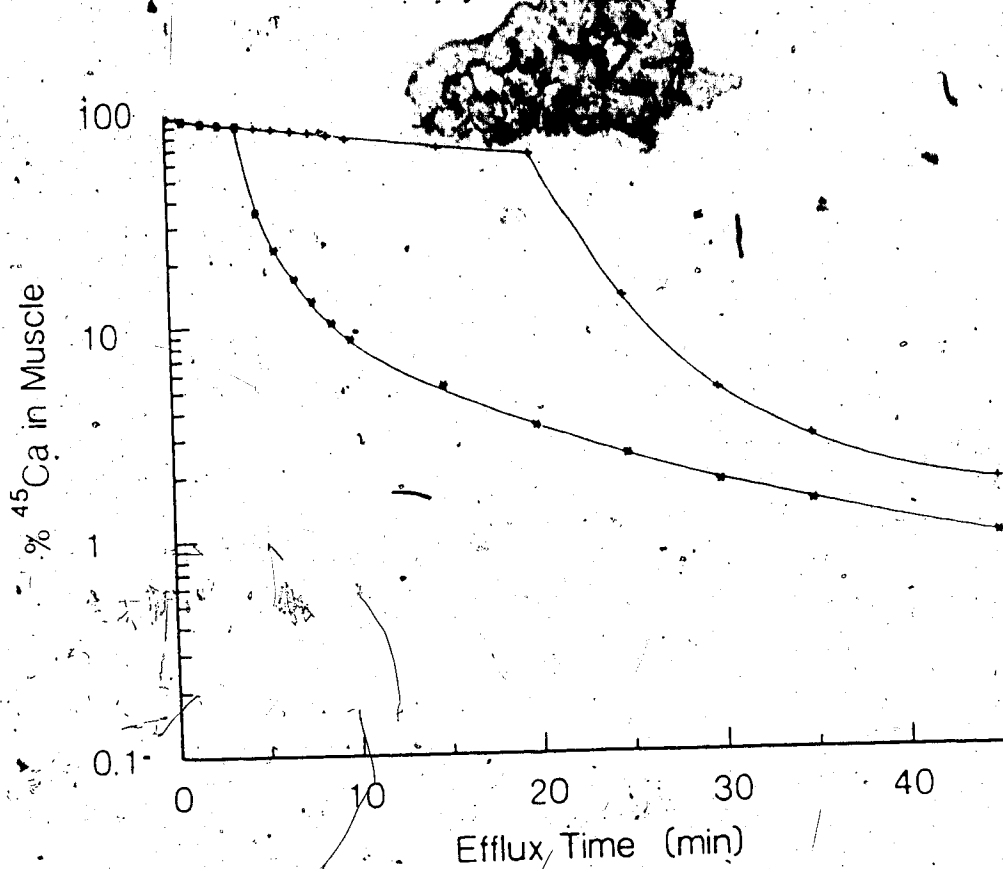
Figure 45. Effects of La^{+++} (2mM) and morphine (10^{-3}M) on ^{45}Ca desaturation curves obtained using a pair of toe muscles from a single frog. The efflux media was Ca^{++}-free Tris Ringer's (CFTR). Its composition for the two toe muscles was as follows:

Efflux Time	Efflux Medium	
(Min)	Muscle 1 (*)	Muscle 2 (+)
0 - 4	CFTR	CFTR
4 - 20	CFTR + La^{+++}	CFTR + Morphine
20 - 45	CFTR + La^{+++} + Morphine	CFTR + La^{+++} + Morphine
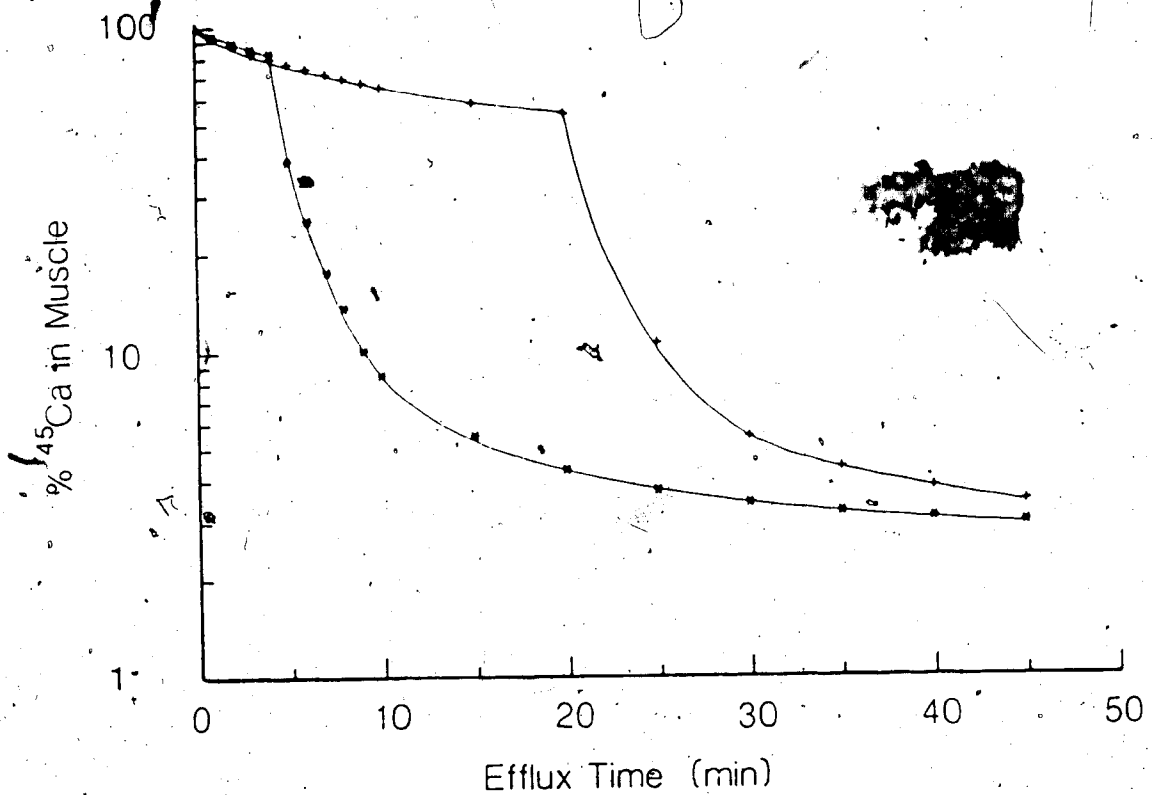
Figure 46. Effects of La⁺⁺⁺ (2mM) and meperidine (10⁻³M) on ⁴⁵Ca desaturation curves obtained from a pair of toe muscles from a single frog. The efflux media composition for the two muscles was as follows:

Efflux Time	Efflux Medium	
(Min)	Muscle 1 (*)	Muscle 2 (+)
0 - 4	CFTR	CFTR
4 - 20	CFTR + La⁺⁺⁺	CFTR + Meperidine
20 - 45	CFTR + La⁺⁺⁺ + Meperidine	CFTR + La⁺⁺⁺ + Meperidine

produced by it (Fig. 47). However, no increase in the ^{45}Ca efflux rate was observed with methadone when it was tested after a prior exposure of the tissue to La^{+++} (2mM) (Fig. 47, muscle 2). Similar results were obtained with 10^{-3} M methadone (n=2 pairs). The results of one of these four experiments is presented in Fig. 47 and 48. These results complemented our earlier observations made under different experimental conditions that methadone (10^{-4} M) caused a small, transient increase in the rate of efflux of ^{45}Ca from the frog toe muscle (Fig. 43).

4.3.3.9 Ca^{++} Uptake Studies.

The muscles used to measure the 10 min uptake of Ca^{++} in Tris Ringer's solution were compared with their counterparts subjected during the later half of their incubation period (5 min) to electrical stimulation (1 Hz.). The mean Ca^{++} uptake by the muscle during the electrical stimulation was roughly twice the uptake seen in the resting condition (Table 15).

Our preliminary Ca^{++} uptake studies with methadone were however, equivocal. A great deal of variability was seen in the results; of the 5 pairs of muscles examined for Ca^{++} uptake following electrical stimulation (300 pulses, 1 Hz.), 3 pairs showed a decreased uptake of Ca^{++} and the other 2 pairs showed
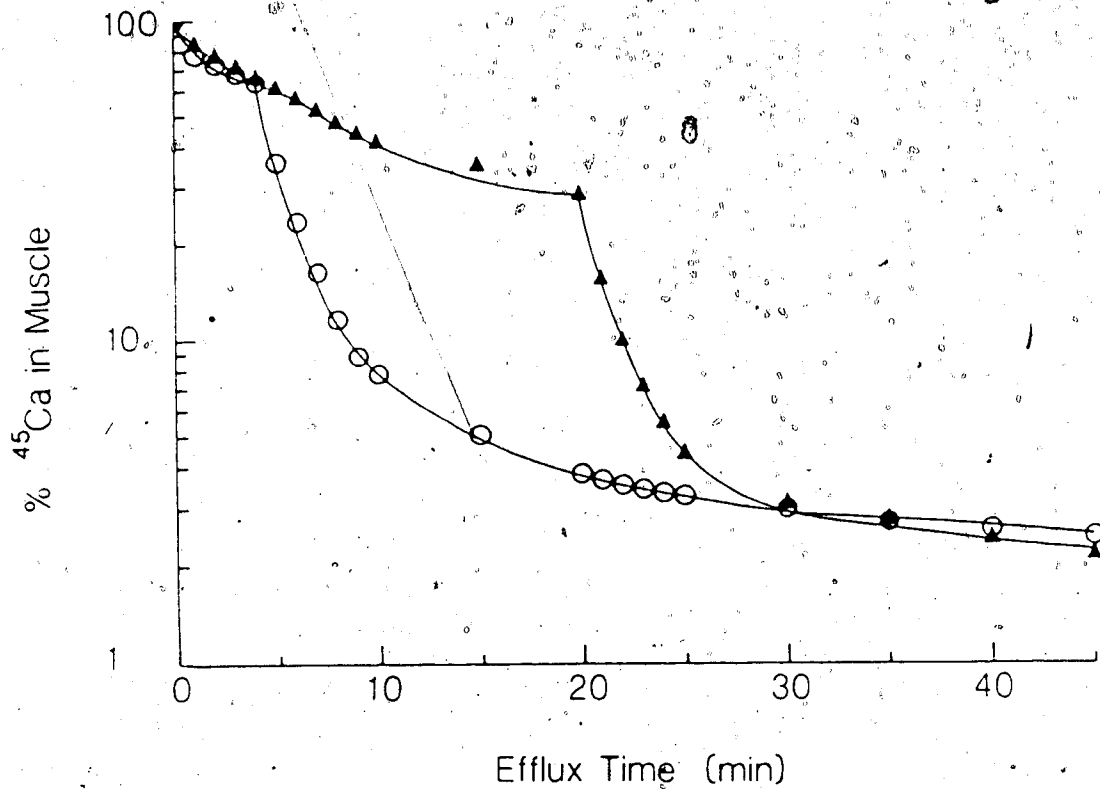
Figure 47: Effects of La^{+++} (2 mM) and methadone (10^{-4} M) on ^{45}Ca desaturation curves obtained from a pair of toe muscles from a single frog. The efflux media composition for the two muscles was as follows:

Efflux Time	Efflux Medium	
(Min)	Muscle 1 (O)	Muscle 2 (▲)
0 - 4	CFTR	CFTR
4 - 20	CFTR + La^{+++}	CFTR + Methadone
20 - 45	CFTR + La^{+++} + Methadone	CFTR + La^{+++} + Methadone
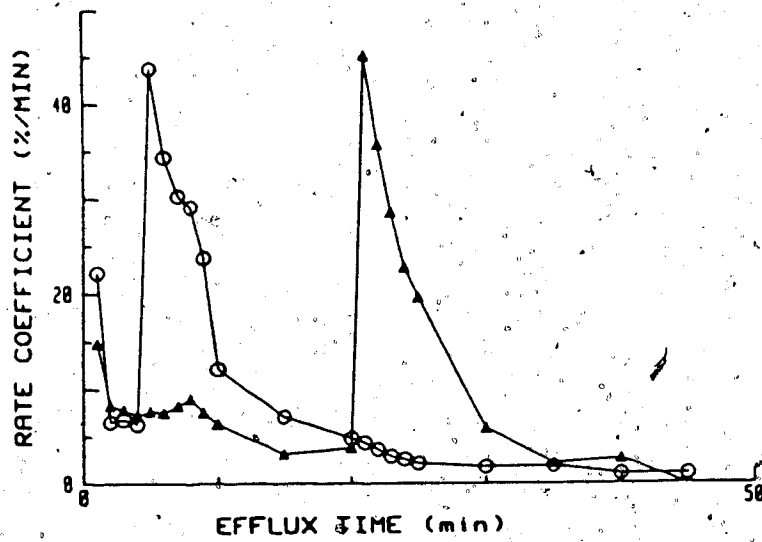
Figure 48.. Efflux rate coefficient curves obtained using
the data in Fig. 47. O, muscle 1; ▲, muscle 2. For
details of the efflux media composition and meanings of
the symbols, see Fig. 47.

TABLE 15.

Ca^{++} Uptake.

Resting Muscle (μM/Kg)	Stimulated Muscle (μM/Kg)	Net Uptake during Stimulation (μM/Kg)	Uptake/ Twitch (nM/Kg)
13.45 ± 5.76	27.65 ± 4.6	14.20 ± 6.95	4.73 ± 2.2

TABLE 16.

Effect of Methadone on total Ca^{++} Uptake.

Control Uptake (μM/Kg)	Uptake in Presence of Methadone (10^{-4} M) (μM/Kg)
20.78 ± 2.4	19.52 ± 3.2

the opposite effect. A comparison of the mean values of $Ca^{\cdot\cdot}$ uptake/twitch under 'control' and under 'test' conditions showed no statistically significant difference between the two (Table 16).

4.3.4 Miscellaneous Experiments

4.3.4.1 Effects of low-$Ca^{\cdot\cdot}$ Ringer's solution on the twitch and the tetanus:

Reducing the extracellular concentration of $Ca^{\cdot\cdot}$ from 1.08 mM to 0.43 mM (a 60% reduction) caused no observable effects either on the twitch or on the tetanus of the frog's toe muscle. Further reductions in the divalent ion's concentration to 0.27 mM (a 75% reduction) or below usually caused the twitches to be potentiated leaving the tetanus unaffected (Fig. 49).

4.3.4.2 Non-opioids and twitch height:

Procaine, a local anesthetic agent lacking agonistic effects on the opioid receptors of frog skeletal muscle fibre membrane [14], and l-hyoscine, a compound that shares some pharmacological properties with meperidine (e.g., spasmolytic activity), were two non-opioids tested separately for their effects on the twitch of the frog toe muscle. The experimental conditions for this study were similar to those used earlier for the opioids.
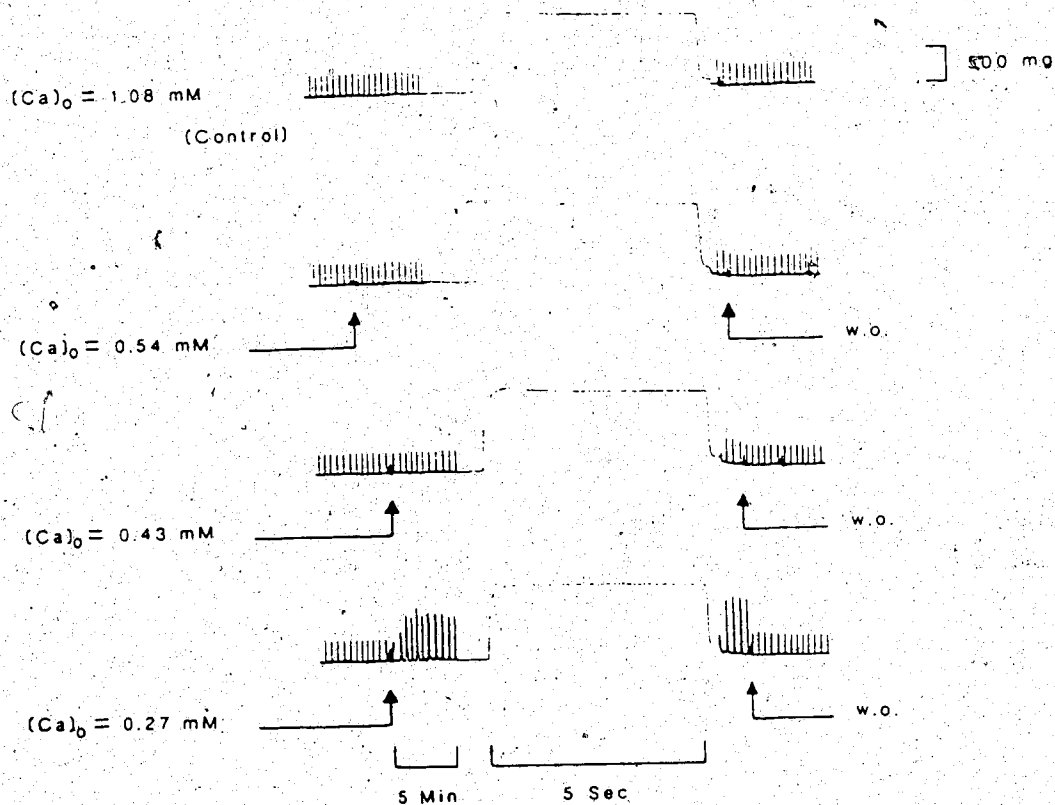
Figure 49. Effect of decreasing extracellular Ca^{++} concentration on the twitch and tetanus of the frog's toe muscle. Twitches were elicited by single electrical shocks applied once every 30 sec. to the muscle; tetanic responses were elicited using electrical pulses at a frequency of 60 Hz for a total period of 5 sec. The above illustration is obtained from one experiment of a set of two repetitions (i.e., N = 2).

Procaine (10^{-7} - 10^{-3} M) produced only a depressant effect on the twitch. This effect became more pronounced with increasing concentrations of the drug (Fig. 50).

On the other hand, l-Hyoscine (0.25×10^{-4} - 8×10^{-3} M) potentiated the twitch in a concentration-dependent manner (Fig. 50). The twitch potentiation by l-hyoscine resembled that produced by opioids, in that the onset of effect following the drug exposure was very rapid (< 30 sec) and the response plateaued quickly (within 3 - 5 min) at each concentration. Prolonged exposures ($> 5 - 10$ min) to higher concentrations ($> 4 \times 10^{-3}$ M) of the drug again resulted in a decrease in the extent of the twitch-potentiation, and also a supramaximal concentration of the drug caused a submaximal effect (Fig. 50).

4.3.4.3 Effects of low-Na⁺ Ringer's solution on the twitch and the tetanus:

Reductions in the extracellular levels of Na⁺ from 111.8 mM to about 44.7 mM (a 40% reduction) caused no significant effects either on the twitch or the tetanus of the isolated frog toe muscle. Further reductions in the Na⁺ concentrations however, markedly depressed the tetanus whilst leaving the twitch relatively unaffected
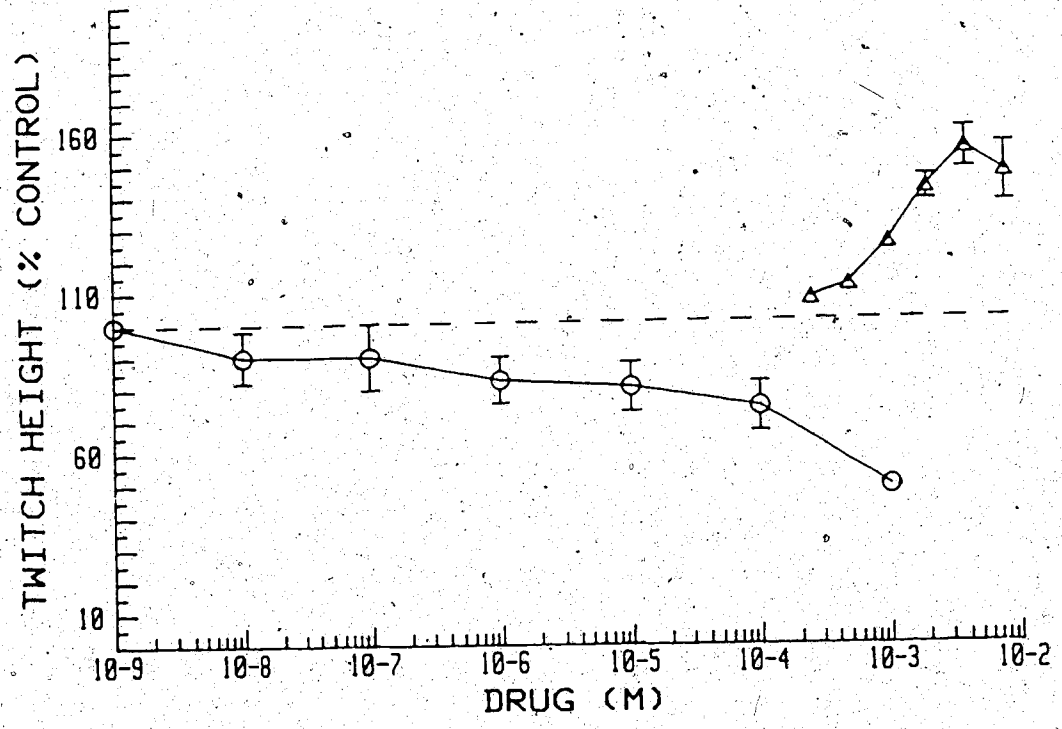
Figure 50. Percent twitch depression or potentiation produced in the isolated frog's toe muscle by procaine or l-hyoscine, respectively. O, procaine, n=4; Δ, l-hyoscine, n=5. Means ± S.E.M. When the S.E.M. is not shown it fell within the symbols. The dotted line represents the control (100%) response.

(Fig. 51). Often (about 50% of the cases) the twitches too were depressed at Na$^+$ concentrations lower than 44.7 mM (Fig. 27), but it was observed that for a given muscle the tetanus was always more sensitive to extracellular Na$^+$ concentration than was the twitch.

4.3.4.4 Effect of procaine on the twitch and the tetanus:

Procaine (10^{-3} M) depressed the twitch of the frog's toe muscle. At a time when the twitch was slightly depressed, the tetanus was completely blocked. In fact, the response to a tetanic stimulation (60 pps) was only a sharp spike lasting less than a fraction of a second. The twitch response immediately following the tetanic response were however, unaffected (Fig. 52).

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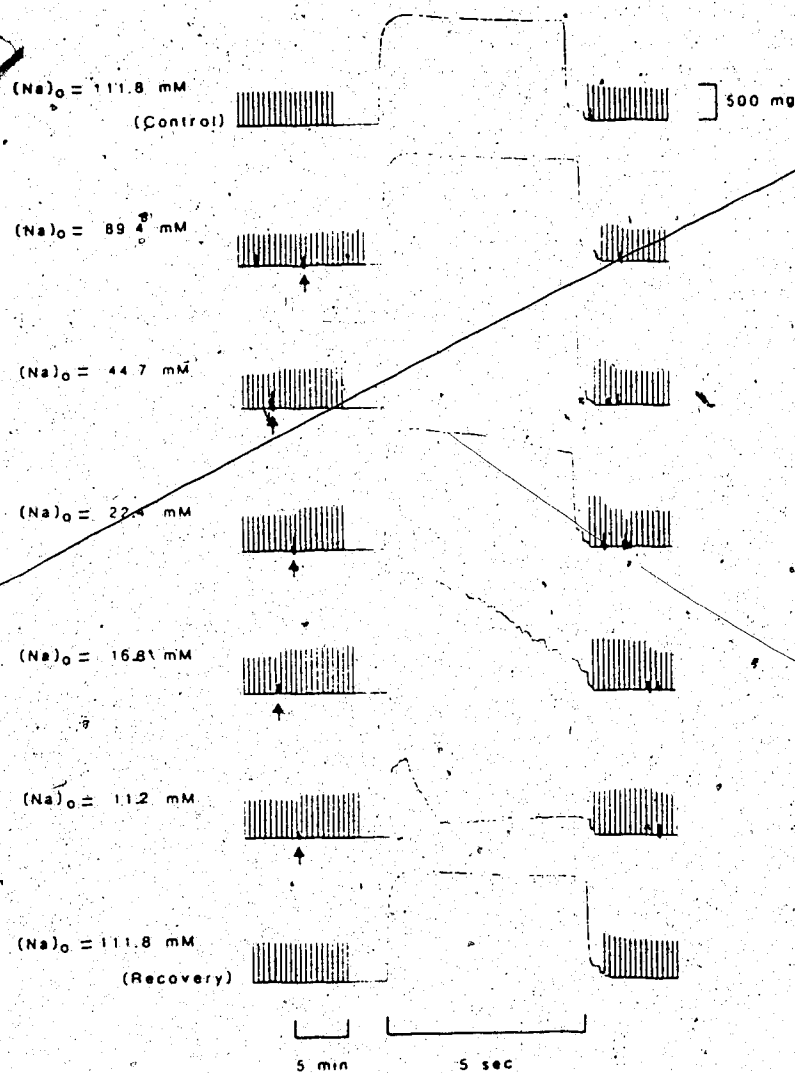
Figure 51. Effect of a decrease in the extracellular Na⁺ concentration on the twitch and the tetanus of frog's toe muscle. Twitch responses were elicited by electrical shocks applied once every 30 sec to the muscle, and the tetanic response was elicited by electrical stimulation at the rate of 60 Hz for a period of 5 sec. The above illustration was obtained from a series of 3 repetitions (i.e., N = 3).
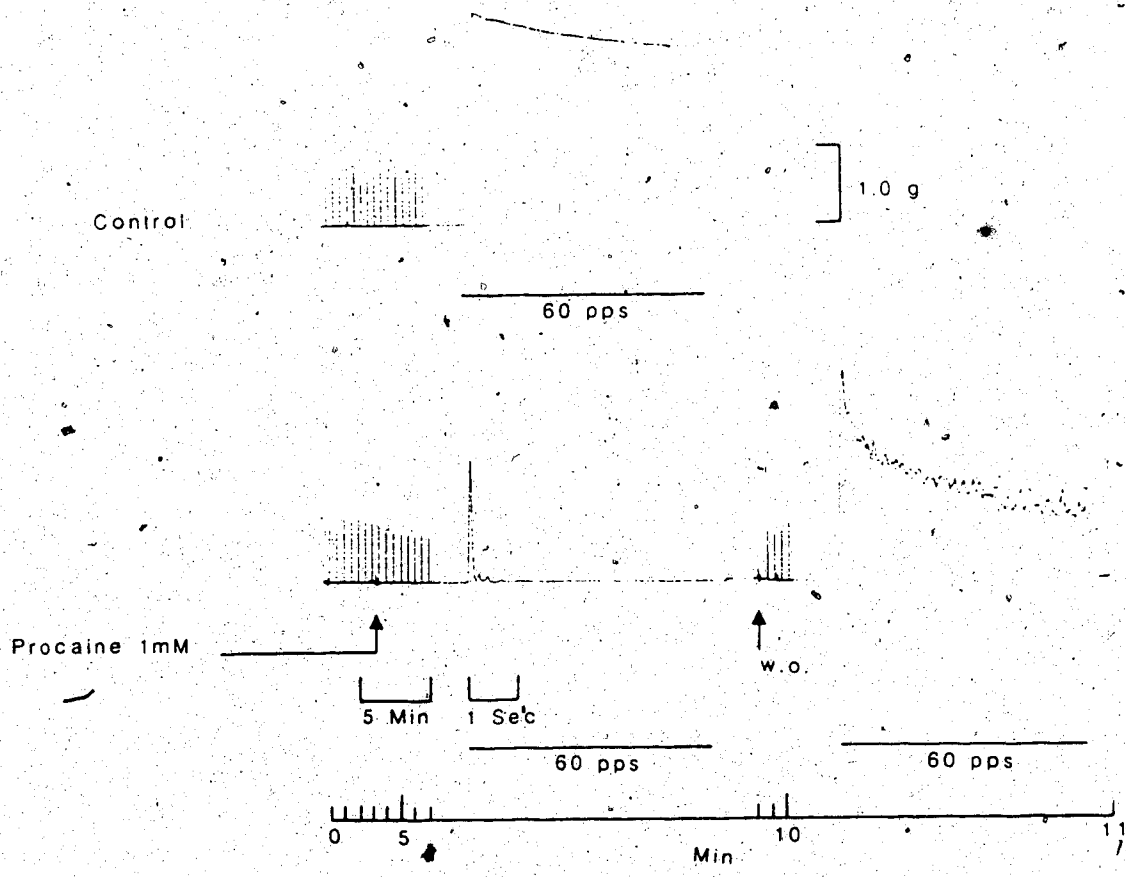
Figure 52. Effect of procaine (10^{-3} M) on the twitch and the tetanus of frog's toe muscle. Twitches were elicited by electrical shocks applied once every 30 sec to the muscle, and the tetanic response was elicited by electrical stimulation at the rate of 60 Hz. The above illustration was taken from a set of 2 repetitions (i.e., N = 2).

5. DISCUSSION

The discovery and description of opioid receptors on frog skeletal muscle fibres by Frank and co-workers [12 - 14] provided a useful *in vitro* system in which the physiological change provided by drug activation of the receptor could be directly measured and studied. These receptors, postulated to be located at or near the intracellular openings of the Na^+ channels, inhibit gNa when activated. The receptors were shown to possess properties that would clearly identify them as functional stereospecific opioid drug receptors. Thus, the receptors were shown to be activated by opioid agonists and very high concentrations of opioid antagonists [12 > 14], and were inhibited by very low concentrations ($<10^{-4}$ M) of opioid antagonists. In addition, they were shown to be stereospecific since only levorphanol, but not its optical isomer dextrorphan, could produce a specific late-occurring depression of gNa sensitive to opioid antagonists [3].

Subsequent to Frank's discovery of opioid receptors in frog's skeletal muscle [12, 13], Durham and Frank [249] observed that meperidine, an opioid agonist, potentiated the twitch in an isolated, curarized and electrically stimulated frog sartorius muscle. This raised the intriguing possibility that activation of opioid receptors in frog's skeletal muscle not only depresses the Na^+ conductance but also potentiates the contractile response of the muscle to a

single electrical shock. These two effects are apparently contradictory since an inhibition of gNa in presence of an opioid agonist should cause an inhibition of the twitch and not a potentiation, as was actually observed. It was therefore of great interest to not only examine the possible involvement of a stereospecific receptor in the mediation of the twitch potentiating effect of the opioid but also to investigate the basic mechanism underlying the above effect.

To establish that the twitch potentiating ability of meperidine, noted by Durham and Frank [249], was not a property unique to that compound, it was necessary to test the effects of a variety of opioids, with diverse chemical structures, on the twitch response of the frog skeletal muscle. The results of such a study indicated that most but not all opioids produce a twitch potentiation, similar to that produced by meperidine [249], in the isolated, curarized and electrically stimulated frog toe muscle. Thus, twitch potentiations were observed with different opioids, each believed to interact predominantly with a particular opioid receptor subtype; e.g., morphine, meperidine, methadone, codeine, fentanyl, levorphanol, - μ-receptor agonists; naloxone, naltrexone, Mr 2096 - μ-receptor antagonists; ketazocine, ethyl ketazocine - κ-receptor agonists; and leu-enkephalin, met-enkaphalin - δ-receptor agonists, all produced this effect. The twitch potentiating abilities of μ agonists or antagonists were,

however, considerably superior to those of either κ- or δ-agonists. On the other hand, etorphine, a potent narcotic analgesic that interacts not only with the μ-receptor, but also with δ- and ϵ- opioid receptors [64], produced no twitch potentiation in the frog's toe muscle. On the contrary, it produced only a depressant effect when exposed to the muscle. Procaine, a non-opioid depressant of muscle excitability did not potentiate the twitch whereas hyoscine, a compound that shares some properties with meperidine (e.g., spasmolytic activity) potentiated the twitch.

The ability of the opioid antagonist naloxone to potentiate the twitch was not surprising because this compound has been demonstrated to be a 'partial agonist' [2]. Therefore, the antagonist if used in high enough concentrations would produce agonist-like effects. Such effects were also shown by earlier studies of Frank [12], and Frazier et al., [263], wherein they observed that the effects of opioid antagonists in high concentrations add on to the effects of opioid agonists. It is quite likely that the antagonists, naltrexone and Mr 2096, also are partial agonists and hence potentiate the twitch responses when used at high concentrations.

Attempts to identify the opioid-induced twitch potentiation with any one of the proposed receptor sub-types met with failure. The opioid-induced twitch potentiation in the frog toe muscle was resistant to blockade by all the

μ-receptor antagonists tested, viz., naloxone, naltrexone, or Mr 2096, suggesting that the effect is not mediated by a receptor akin to the μ-receptor described in the guinea pig ileum [57]. A small antagonism was observed when naloxone (5×10^{-6} or 2×10^{-5} M but not 1×10^{-5} M) was employed to counteract the twitch potentiation caused by methadone (Fig. 25). However, naloxone when tested alone also caused a depression of the 'control' twitch responses at low concentrations (i.e., $<5 \times 10^{-5}$ M) (Table 2; Fig. 13) Therefore, the small antagonisms seen with naloxone were due to its direct depressant action on the twitch resulting in addition of opposite responses and not due to any pharmacological antagonism. When high concentrations of the above antagonists were tested in combination with the agonist, they produced additive effects which were similar to the additive effects observed by Frank [12], and Frazier et al., [263] discussed in the preceding paragraph. Phenoxybenzamine, a compound reported to cause a long-lasting inactivation of the opioid receptors of the μ- and δ-type [62] also was unsuccessful in antagonizing the twitch potentiation caused by methadone. κ-agonists (e.g., (-)ketazocine or (-)ethyl ketazocine) as well as δ-agonists (e.g., the enkephalins) potentiate the twitch in this preparation, but the lack of any specific κ- or δ-antagonist coupled with the relative resistance of the κ- and δ receptors to the actions of naloxone [57, 264]

preclude the elimination of the involvement of any of the above receptor subtypes in this preparation. But even so there was no evidence of a selectivity necessary to identify one of the receptor subtypes.

The analgesic activity like most of the actions of opioid drugs is highly stereospecific, with almost all the activity residing in those isomers with a configuration analogous to that of D(-) morphine. If the twitch potentiation studied here involved a similar stereospecific opioid receptor, then the D(-) isomers should have had much higher potencies than the corresponding L(+) isomers. However, this was not the case. For example, we found that levorphanol, a potent opioid agonist that has the D(-) configuration and dextrorphan, the analgesically inactive L(+) isomer did not exhibit a statistically significant difference in their relative potencies in potentiating the twitch. Moreover, even though we did find that a racemic mixture of morphine was more potent in potentiating the twitch then the analgesically inactive isomer, (+) morphine, the reliability of this result is questionable because the experiment could be repeated only once (n=2) owing to the very small amount of the (+) morphine available to us.

Our results therefore show that the twitch potentiating effect of the opioids is not mediated by a stereospecific opioid receptor of the μ-type. Thus, this effect could be a consequence of the activation of nonstereospecific and

naloxone-resistant opioid receptors. Similar findings have been reported by Miranda *et al*., [76], and by Jacquet [265] who have attributed the potentiation of the electrically stimulated contractions of the rat *vas deferens* to activation of such nonstereospecific and naloxone-resistant receptors. The existence of such receptors has also been postulated by Nakatsu *et al*., [266] who have noted that the enkephalin-induced relaxation in the isolated rat ileum is resistant to blockade by naloxone. Similar receptors also seem to exist in the periaqueductal gray region of the central nervous system of rats where they are believed to mediate the hyperreactive effects of morphine [267]. It is possible that such opioid-sensitive but naloxone-insensitive receptors also exist elsewhere and may be responsible for phenomena such as analgesia currently classified as being 'non-endorphin' [268, 269].

It was previously shown that opioid agonists depress action potential production by two mechanisms of action; an initial nonspecific inhibition of both' gNa and gK and a specific inhibition of gNa. Only the latter action involves a stereospecific opioid receptor [13, 14]. In the present study when morphine and fentanyl were tested at high concentrations (10^{-3} M) and for prolonged periods of time they produced an initial twitch potentiation followed by a progressive decrease in twitch height. These depressant effects on the twitch were found to be naloxone-resistant

and at least in the case of morphine, the one opioid examined in detail [14], was found to correspond in time to the nonspecific changes in the electrical properties of the frog skeletal muscle fibre membrane. This suggests that under the experimental conditions in the present study, the opioids produce a depression of twitch height by causing a nonspecific inhibition of the active electric characteristics of the membrane.

Most of the experiments designed to investigate the twitch potentiating effects of opioids were carried out using methadone, morphine or meperidine because all these compounds produced sizeable twitch potentiations and because they were available in sufficient amounts. In this part of the study it was assumed that the opioids that produced twitch potentiations, did so by a common mechanism, although minor differences in actions were noted amongst the various opioids tested. This assumption is considered valid because the qualitative effects of a variety of opioids on the twitch of the isolated, curarized and electrically stimulated frog toe muscle are almost identical. Experiments that were considered crucial to our interpretations were performed with more than one opioid and the results thus obtained were compared.

Augmented contractile force of a muscle in the presence of a drug could be a consequence of one or more of several possible changes in the muscle. For instance, a drug could

cause a recruitment of additional muscle fibres in a muscle. Thus, the increased number of active motor units would produce increased contraction by activation of more muscle fibres. Also, a given electrical stimulus that is submaximal for the muscle in drug-free condition could be maximal in presence of the drug and this alone could cause augmentation of the muscle response by merely activating all instead of only a part of its fibres. That such effects played no role in our experiments was ensured by using d-tubocurarine in the bathing medium thereby eliminating neural and junctional effects, and by using electrical shocks that were sufficiently intense to make all muscle fibres respond maximally.

Another mechanism by which the contractile force of a muscle can be augmented is by repetitive discharge in individual muscle fibres. Such an effect can be caused by veratrinic agents [270], or even with an anionic potentiator under certain conditions [271, 272]. In these cases, exposure of the muscle to the agent causes each muscle fibre to fire several action potentials instead of a single one when stimulated with a single electrical shock. Repetitive discharge in a single muscle fibre would increase tension by summation of successive twitches. Thus, in these cases, the response to the single shock is not a potentiated twitch but a tetanus. However, as shown by our intracellular recording experiments, the opioids tested, viz., dextrorphan,

naloxone, etorphine, or methadone do not produce a veratridine-like effect, i.e., they do not cause repetitive firing of action potentials in response to a single shock. Therefore the responses observed with the opioids are not tetani but potentiated twitches.

The time taken by an opioid to produce its peak twitch potentiating effect, at any particular concentration, was found to be independent of the muscle stimulation, i.e., the amount of time needed by the muscle to exhibit its maximal response to an opioid was the same irrespective of whether the muscle was periodically stimulated during that time period or was quiescent (Fig. 14). This suggests that the peak effect of an opioid is solely a function of the time taken by the compound to attain its optimal concentration at its site of action.

A sizeable twitch potentiating concentration of methadone, viz., 5×10^{-5} M, was found to increase the range of pulse intervals at which the responses were fused from 0.5 to 10 msec to 0.5 to 30 or 40 msec, showing that the successive shocks applied to the treated muscle can be as much as 30 or 40 msec apart, instead of the normal of about 10 msec and yet produce a fused response. Therefore, the reduction in the fusion frequency by the opioid from about 100 per sec to about 67 or 50 per second suggests that the active state plateau produced by each shock lasts longer in the presence of methadone. That this effect was not seen in

the presence of other opioids, viz., morphine or meperidine was possibly due to the use of low concentrations of the compounds that produced much smaller twitch potentiations than did methadone. Additionally, methadone did not change the relationship existing between the peak tension and the time to peak tension of the twitch; it only moved it to higher values of tension (Fig. 21). This again indicated that the opioid prolonged the duration of the active state of this muscle; this phenomenon however, was not explored any further.

Twitch potentiation in a skeletal muscle fibre might be produced in a variety of ways:

- A potentiating agent could increase the influx of Ca^{++} during stimulation and thereby enhance the degree of activation of the contractile apparatus, as has been reported to occur for NO_3^- [260].

- A compound could act on the sarcoplasmic reticulum membrane and either induce an increased release of Ca^{++} or inhibit the reuptake of Ca^{++}, thereby causing an elevation in the myoplasmic concentration of ionized (or 'free') calcium. This would produce an augmented contractile response. Compounds believed to act in this way include: caffeine, 4-aminopyridine, acetaldehyde, pentobarbital, and diethyl stilbestrol [273 - 277].

- A drug could influence the contractile properties of

skeletal muscles by altering the Ca^{++} sensitivity of the contractile proteins. Such a mechanism has been proposed to explain the twitch potentiating effects of physostigmine [278].

- Chemical agents like NO_3^- or Zn^{++} have been postulated to potentiate the twitch by their effects on the electrical properties of the muscle fibre membrane. Sandow and co-workers [245] have suggested that the decrease in the mechanical threshold produced by NO_3^- or the increase in the action potential duration caused by Zn^{++} is responsible for the augmented mechanical output of the twitch produced by the two ions. The above mentioned changes in the membrane electrical properties were postulated to increase the mechanically effective period of the action potential, which they believed was the main factor determining the duration of the active state and hence the tension output of the twitch.

- The twitch potentiating effects of low concentrations of some multivalent cations (e.g., Co^{++}) has been explained on the basis of the 'trigger Ca^{++} hypothesis.' This hypothesis suggests that there are superficial binding sites for Ca^{++} on the luminal surface of the t-tubular membrane and that occupation of these sites by Ca^{++} increases the binding (or affinity) of 'trigger' Ca^{++} to this membrane. When the

luminal Ca^{++} are displaced from their sites either by multivalent cations or by a lowered extracellular concentration of Ca^{++}, each action potential releases more 'trigger' Ca^{++} into the triadic junction and the twitch is potentiated [225, 226, 243, 217, 221].

From the above list of some of the proposed mechanisms for twitch potentiation by different chemical agents, it is obvious that the 1st step in the elucidation of the mechanism of twitch potentiation by any agent is a localization of its site of action.

The twitch potentiating effect of the opioids has a very rapid onset of action. The effect is observable within 30 sec of drug-exposure suggesting that these compounds act at sites readily accessible to the extracellular-fluid. This conclusion is reinforced by the observation that the opioid-induced twitch potentiation can be rapidly reversed on restoration of the muscles to drug-free Ringer's solution even when the muscles had previously been exposed to the drug for as long as 20 min (Fig. 9). Such prolonged exposures would undoubtedly cause penetration of some of the drug into the fibres but the rapid reversal kinetics suggest that the intracellularly trapped drug is unable to cause twitch potentiation. This, therefore implies that the reversal of the effect is a function of the rate at which the drug diffuses away from the muscle fibre membrane. Furthermore, reports by Frank and co-workers [13, 14] have

indicated that opioids like meperidine, morphine, or methadone penetrate into the muscle sarcoplasm rather sluggishly. The muscles need to be incubated with relatively low opioid concentrations for periods in excess of 2 - 3 hrs before a sufficiently high concentration of the drug can be built up intracellularly in order to activate the opioid receptors located on the inner surface of the plasma membrane. However, at the same concentrations the 'local anesthetic-like' effects of the opioids, were found to commence immediately, suggesting that the opioids either act at the outer surface of the muscle fibre membrane or dissolve in the membrane itself to produce this effect. These observations too argue against the possibility that the opioids rapidly penetrate into the muscle fibres and act at some intracellular site such as the sarcoplasmic reticulum or the muscle contractile proteins to produce their twitch potentiating effects.

Another feature of the opioid action that argued against an intracellular site of action was the rapid 'plateauing' effect seen with submaximal concentrations of the drugs. In these experiments it was observed that the opioids quickly produced a peak effect at a given concentration and the twitch remained at that level as long as the muscle remained exposed to the drug. The response never increased in magnitude with increased duration of drug exposure to match the magnitude of a response exhibited with

a shorter exposure to a higher concentration of the drug. This clearly would not have been the case had the site of action of the opioid been intracellular. In such a situation, the response would be expected to increase in magnitude with time due to a build up of the drug concentration at its intracellular site of action as happens when a skeletal muscle is exposed to a submaximal concentration of an intracellularly acting compound like caffeine. Thus, it is suggested from the foregoing discussion that opioids produce their twitch potentiating effects by acting on the muscle fibre membrane.
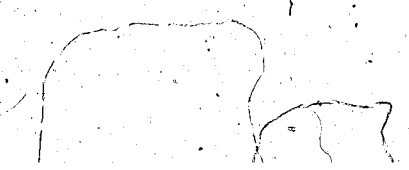
Ca^{++} is known to be of paramount importance in the contractile process of muscle. Our experiments discussed earlier, implied that opioids prolong the active state of the muscle by increasing the availability of Ca^{++} to the contractile apparatus. It was therefore of interest to see if opioids did this by promoting the influx of Ca^{++} through the slow Ca^{++} channels (present in the t-tubular membranes) during depolarization of the muscle fibre membrane. It has been shown that K^{+}-depolarization-induced contractures use extracellular Ca^{++} [243] and that the influx of Ca^{++} into the muscle fibre takes place through voltage-dependent Ca^{++} channels sensitive to organic Ca^{++} channel blockers like D-600 [279]. Therefore, in order to determine if opioids promote an increased influx of Ca^{++} through the above channels, the effects of methadone on K^{+}-induced

contractures were studied. Our results showed that methadone (Fig. 33) markedly depressed the K^+-contracture response whilst potentiating the twitch response in the same muscle. This clearly showed that opioids do not increase the influx of Ca^{++} through the slow Ca^{++} channels. In fact, the K^+-contracture experiments implied that the opioid actually decreased the influx of Ca^{++} during the contracture. Such Ca^{++}-antagonistic effects of opioids have also been observed by several other investigators in different experimental systems [100, 280, 281].

The conclusion that opioids do not use the slow Ca^{++} channel in producing their twitch potentiating effect was reinforced by the results obtained with the use of D-600. D-600 has been extensively employed as a slow Ca^{++} channel blocker in studies on cardiac and smooth muscle function [282]. Frank [279] showed that D-600 (10^{-6} M) blocks K^+-induced contractures in frog's toe muscle, indicating that the voltage-dependent Ca^{++} channels in vertebrate skeletal muscles are sensitive to D-600. The same or a higher concentration of the compound, viz., 3×10^{-6} M, when tested, were found to have no antagonistic effects on the twitch potentiations produced by methadone, meperidine or morphine. Similar results were obtained even in muscles in which the K^+-contractures were initially almost completely blocked by the Ca^{++} antagonist. During this time, neither the 'control' twitches nor the potentiated twitches were

affected. These experiments with D-600, therefore, show unequivocally that opioids do not use the slow Ca^{++} channels in producing a potentiated twitch response. This conclusion also received support from the Ca^{++} uptake experiments: in which no additional uptake of Ca^{++} was noticed in the presence of methadone (Table 15). The latter experiments were preliminary, however, and the results obtained in the different experiments were not consistent.

One treatment that could antagonize the twitch potentiation by opioids was an elevation of the extracellular concentration of Ca^{++} from 1.08 to 8.64 mM. This increase in the Ca^{++} concentration did reduce the 'control' twitch size initially but the response, more often than not, recovered to 'control' levels in about 40 min. Frank [225] explained this phenomenon of twitch depression in the presence of high extracellular levels of Ca^{++} by stating that the amount of the membrane bound 'trigger' Ca^{++} released is controlled by the degree of saturation of the superficial membrane sites by Ca^{++} in a roughly inverse manner, i.e., the higher the concentration of Ca^{++} in the extracellular fluid, the higher is the saturation of the superficial Ca^{++} binding sites on the membrane and therefore less 'trigger' Ca^{++} is released per stimulus and the reverse occurs when the amount of superficially bound Ca^{++} is reduced. Therefore, the antagonistic effect of the high extracellular concentration of Ca^{++} might be explained by

one of several possible mechanisms. It is possible that opioids are causing a displacement of the superficially bound Ca^{++} which presumably would decrease the 'hold' of the membrane bound 'trigger' Ca^{++} and consequently a larger amount of the ion would be released per stimulus. If this were the case, then an elevation of the extracellular Ca^{++} concentration would antagonize the opioid effects by not only replenishing the sites depleted of Ca^{++} by the opioid but also by additionally saturating superficial sites and rendering the 'trigger' Ca^{++} less likely to be released during the stimulation. Alternatively, it is possible that the opioids are somehow directly rendering the pool of 'trigger' Ca^{++} more labile thereby enhancing their release during stimulation and that elevation of the extracellular concentration of Ca^{++} simply renders that pool of Ca^{++} less labile by the saturation mechanism just discussed.

Another possibility is that the high-Ca^{++} concentration, in the bath, which would elevate the myoplasmic level of Ca^{++} [113, 260], antagonizes the opioid effect intracellularly by some unknown mechanism. This seems unlikely because, as already discussed, the opioid effect is rapidly reversible when the drug is removed from the bathing solution.

The most likely possibility seemed to be that opioids were displacing the superficially bound membrane Ca^{++}. To test this possibility, ^{45}Ca desaturation studies were

conducted. In these experiments, La^{+++}, an ion that displaces and replaces Ca^{++} at superficial binding sites on frog sartorius muscle [283] and on many other muscle types [284 - 286] was used as a tool to indicate the presence of a detectable pool of superficially bound Ca^{++} under experimental conditions similar to those used for testing the effects of three opioids. The La^{+++} effects also served as standards for comparison with the effects produced by these opioids on the superficially bound membrane Ca^{++} in the one instance where an effect was found (i.e., methadone). Finally, the use of this ion helped to identify superficial Ca^{++} binding sites sensitive to La^{+++} displacement. La^{+++} was particularly suitable for the above purposes because it does not penetrate the skeletal muscle sarcolemma unless the muscle is damaged [287 - 289] implying that its site of action is on the sarcolemma [287].

The addition of La^{+++} (2mM) to muscles previously incubated in Tris Ringer's solution containing ^{45}Ca and washed out in Ca^{++}-free Tris Ringer's solution induced a detectable transient increase in ^{45}Ca efflux (Fig. 41). Such a transient increase is believed to indicate displacement of the radioactive tracer from cell surface membrane sites representing a limited portion of the total ^{45}Ca content of the tissue [290]. During the wash with La^{+++} following an incubation lasting 3-4 hrs in Tris Ringer's solution containing ^{45}Ca, a point in time is

reached (usually between 2 and 3 hr of efflux) where the percentage of ^{45}Ca remaining in the La^{+++} test and 'control' tissues are identical. Before this crossover point, the La^{+++} 'test' muscles contained less ^{45}Ca than controls (presumably because they have lost more of the ^{45}Ca bound to the extracellular surface of the sarcolemma and have less total ^{45}Ca content despite a higher intracellular ^{45}Ca concentration). However, after this point, the experimental tissues contain more ^{45}Ca than controls (presumably because they now retain more intracellular ^{45}Ca, and both tissues have been depleted of their superficially bound ^{45}Ca stores). The results with La^{+++} support previous findings of Weiss [283] suggesting the presence of Ca^{++} at superficial anionic binding sites exposed to the extracellular fluid which can be displaced by La^{+++}. In addition, La^{+++} diminished if not abolished the efflux of intracellular Ca^{++}.

The addition of methadone 10^{-4}M to muscles previously incubated in normal Ringer's solution containing ^{45}Ca and washed out in a Ca^{++}-free Ringer's solution also induced an initial increase in ^{45}Ca efflux (Fig. 43). This increased ^{45}Ca efflux rate appeared to continue longer with methadone (Fig. 44) than it did with La^{+++} (Fig. 42). Possibly this was due to an additional effect of methadone on the efflux of intracellular stores of ^{45}Ca. However, under similar experimental conditions neither morphine (10^{-4} or 10^{-3} M)

nor meperidine $(10^{-4}$ M) had an effect on the ^{45}Ca desaturation rates.

These variable effects of the opioids prompted us to modify the experimental conditions in order to increase the sensitivity of our technique for detecting movements of superficially bound Ca^{++}. Under the modified experimental protocol the tissues were soaked in a Ca^{++}-free medium for several hours to deplete their Ca^{++} stores and then they were exposed briefly (30 min) to ^{45}Ca present in the same Ca^{++}-free medium. Since the superficial stores were depleted of their Ca^{++} content under these experimental conditions, there would presumably be a greater number of binding sites available for ^{45}Ca resulting in a higher specific activity of the Ca^{++} bound to these superficial membrane sites. This was expected to make our tests more sensitive to membrane ^{45}Ca displacement and thereby make it possible to detect displacements of a smaller proportion of the ^{45}Ca bound to these superficial sites.

Our results with La^{+++} indicate that our experimental objective had been achieved. Thus, for example, when the muscle was exposed to ^{45}Ca for 3-4 hr in normal Ringer's solution before testing (Fig. 41), a 5 min exposure to La^{+++} resulted in an 81% loss of the tracer from the muscle but even the 'control' muscle lost 75% of the label at 5 min. The difference while significant, was small. In contrast, with the modified technique (Fig. 45 and 46) a 5 min

exposure to La^{+++} produced about a 90% loss in the ^{45}Ca from the muscles whereas the other muscles (with morphine or meperidine) had lost only about 20-30% of the label. In addition after 45 min of efflux in the latter type of experiments the muscles had lost about 98 or 99% of their starting ^{45}Ca content whereas with the initial procedure (Fig. 41), even with La^{+++}, at 45 min the muscles still contained more than 10% of the starting content and the ^{45}Ca content was only down to 2-3% after 4 hr of efflux. It appeared that with the modified technique almost all of the ^{45}Ca had been taken up by superficial binding sites and this Ca^{++} was readily displaced by La^{+++}.

With our initial procedure, which is more like the procedure used by most previous workers, more of the ^{45}Ca had entered intracellular sites and a large proportion of the ^{45}Ca remained in the extracellular fluid. The latter point is indicated by the 75% loss of ^{45}Ca during the first 5 min of efflux in 'control' muscles using the initial procedure. In contrast with the modified technique, a 5 min exposure to morphine or meperidine which did not displace membrane ^{45}Ca, only 20 to 30% of the ^{45}Ca was lost from the muscles.

In the latter type of experiments (Figs. 45 - 47) we used La^{+++} (2mM) as a standard for comparison of the effects of the opioids on superficial Ca^{++} binding sites. However, even under these modified, more sensitive experimental

conditions, neither morphine (10^{-3} M) nor meperidine (10^{-3} M) had any detectable effect on the efflux rate of ^{45}Ca. Therefore it must be concluded that these compounds do not displace the superficially bound Ca^{++} from frog toe muscles. On the other hand, methadone (10^{-4} or 10^{-3} M) did cause a slight but significant increase in the efflux rate of ^{45}Ca (Fig. 48) as observed earlier under different experimental conditions (Fig. 44). It is apparent that the action of this opioid on the rate of ^{45}Ca efflux is much weaker and develops more slowly than that observed with La^{+++} (Fig. 48). Since, as already discussed, all or almost all of the ^{45}Ca is on a superficial binding site when using our modified technique, the ability of methadone to cause some increase in the efflux rate using this technique, suggests that this opioid has some effect at an easily accessible and presumably superficial site on the muscle fiber membrane. This conclusion was further supported by the observation that methadone did not produce an increase in the rate of ^{45}Ca efflux when tested after La^{+++} (Fig. 48, muscle 1) indicating that both these agents act on the same pool of Ca^{++}; viz., the La^{+++}-accessible, superficially bound membrane Ca^{++}. Therefore methadone does cause a release of Ca^{++} from the superficial membrane sites. This result suggests that methadone may prevent the rebinding of Ca^{++} spontaneously released from these superficial sites into a Ca^{++}-free solution.

These experimental results do not support our original hypothesis that opioids potentiate twitches in curarised frog toe muscles by displacing the superficially bound membrane Ca^{++} from the muscles. Morphine (10^{-4} or 10^{-3} M) or meperidine (10^{-4} or 10^{-3} M) at concentrations that produced good twitch potentiations, did not displace any ^{45}Ca from superficial sites on the muscle fibre membrane. Although methadone produced a slight displacement of ^{45}Ca from this pool, the contribution of such a displacement to the opioid effect on the twitch is not clear.

The findings that opioids potentiate the twitch by an effect on the muscle fibre membrane but not by displacing the superficially bound membrane Ca^{++} persuaded me to examine the effect of these compounds on the electrical properties of the membrane. The opioid effects were examined on the resting membrane potential, action potential amplitude, the maximum rate of rise/fall of the action potential and on the action potential duration (measured at -40 mV). The action potential duration was measured at -40 mV since the full tension of a single muscle fibre was reported to be attained by a depolarization to this level [291]. The results with the intracellular recording experiments clearly showed that while high concentrations of the opioids dextrorphan or naloxone caused depressions in the action potential's maximal rates of rise/fall and produced a prolongation of the action potential duration, no

such changes were observed with lower concentrations of the compounds, especially when tested within a 5 min exposure of the muscle to the opioids. These lower concentrations of the opioids, however, did produce sizeable twitch potentiations in the frog toe muscle (Fig. 26; Table 2). These results clearly demonstrated a lack of correlation between the manifestation of the twitch potentiating effect and the electrical changes. The only parameter of the action potential that has been postulated to regulate the amplitude of the twitch is the action potential duration [292]. It was proposed that a prolongation of the action potential duration causes the muscle to remain in the active state for a longer duration of time and this causes an augmentation of the mechanical response of the muscle. Though, we did find that all the opioids tested, viz., dextrorphan, naloxone or methadone, produce a marked prolongation of the action potential duration, they produced this effect only at high concentrations or after prolonged incubations with lower drug concentrations. There was a clear lack of demonstrable correlation between the concentrations required for the manifestation of the twitch potentiating effect and those required for the prolongation of the action potential duration, within 5 min of drug exposure (Fig. 26). Moreover, etorphine, a compound that did not potentiate the twitch also produced identical changes in the various electrical parameters of the muscle

fibre membrane tested including the action potential duration. This demonstrated unequivocally that no causal relationship exists between the changes in the electrical properties of the membrane produced by the opioids and their twitch potentiating abilities especially at lower concentrations. However, a contribution of the prolonged action potential duration, observed at higher drug concentrations to the twitch potentiating effect of the drugs cannot be ruled out completely.

The conclusion that the electrical changes do not by themselves cause the twitch potentiation was reinforced by the observation that elevated extracellular Ca^{++} concentrations (8.64 or 10.08 mM), which antagonized the twitch potentiation by opioids, does not antagonize the changes in the electrical properties of membrane produced by methadone (Fig. 34 - 38).

Although increasing the extracellular Ca^{++} concentration antagonized the opioid effects on the twitch, no change of this drug effect was observed following lowering of the Ca^{++} concentration down to 0.216 mM (an 80% reduction). Further reductions in the Ca^{++} concentration increased the excitability of the muscle and caused it to fire repeatedly to single electrical shocks resulting in the elicitation of tetanic responses.

Antagonism of the opioid effect on the twitch also was observed in the presence of contracture-producing

concentrations of caffeine or of the Ca^{++} ionophore, A23187 (Fig. 39,40; Table 14). It is well known that caffeine can cause contraction of skeletal muscle without depolarization [293 - 295] through the release of Ca^{++} from the sarcoplasmic reticulum [240]. The antagonism of opioid effects by caffeine suggested several possibilities, viz., that the increase in the myoplasmic level of Ca^{++} by the compound might antagonize the opioid twitch potentiation, or that it might produce ultrastructural damage to the myofibrils of the muscle fibres and thereby impair the ability of the muscle to exhibit its usual response to the opioid, or that it might cause a substantial reduction in the Ca^{++} content of the sarcoplasmic reticulum thereby preventing the opioid from ultimately inducing the release of any additional Ca^{++} from this st during the stimulation. It has been proposed that elevation of intracellular Ca^{++} concentration promotes the activity of proteases which cause rapid myofilament degradation [296]. That such a process might be occurring in our experiments is suggested by the observation that the twitch height, one hour after washout of caffeine (3×10^{-3} M) from the muscle, is considerably reduced although the muscle now responds to the opioid in its usual manner (Fig. 39, 40). The latter observation suggests that an inhibition of the opioid response in a caffeine treated muscle is not the consequence of ultrastructural damage caused by caffeine but is due

either to a genuine antagonism of the opioid effect by elevated intracellular concentration of Ca^{++} or to a reduction in the Ca^{++} stores of the sarcoplasmic reticulum available for release. A similar argument for the antagonistic effect of the Ca^{++} ionophore A-23187, may be applied since even this compound has been reported to act in a manner analogous to caffeine. It was shown that treatment of frog [297] or mouse [298] skeletal muscle with the divalent cation ionophore (5 μg/ml) causes major ultrastructural damage, with dissolution of the myofilaments. It was also shown that the ionophore acts primarily at the sarcoplasmic reticulum, causing the release of stored Ca^{++} and that the consequent rise in $[Ca^{++}]_i$ promoted (directly or indirectly) the activity of proteases which in turn degraded the myofilaments.

Thus two drugs which are known to raise the intracellular levels of Ca^{++} antagonize the twitch potentiation produced by the opioids. However, they produce this antagonism only at concentrations much higher than required to antagonize the effects of opioids or the intracellularly located stereospecific receptors which modify the activity of the Na^+ channels [113]. Moreover, these high concentrations of caffeine and A23187 required also produced permanent reductions in the twitch producing ability of the muscle fibres. This suggests that they were producing permanent structural changes in the muscles

modifying e-c coupling. However this line of study was not pursued further.

The peak twitch potentiation produced by an opioid was found to decrease in a graded manner with graded reductions in the concentration of the extracellular Na^+ (Fig. 28). In fact, with an extracellular Na^+ concentration of about 44.7 mM, the opioids did not produce any augmentation of the twitch response but rather depressed the twitch. Such a phenomenon was observed even with a concentration of an opioid that produced minimal twitch potentiations (Fig. 29). In addition, this effect could be instantaneously reversed by restoring the Na^+ concentration to its initial level. These findings were intriguing because at the outset they suggested an existence of a reciprocal relationship between the concentrations of Na^+ and Ca^{++} in regulating the extent of the potentiation. It may be recalled that elevation of extracellular Ca^{++} beyond a certain level also caused an antagonism of the opioid-effect on the twitch, as discussed earlier.

This reduced concentration of Na^+, however, caused no change or only a slight potentiation of the twitch response. Similar twitch potentiations with low concentrations of Na^+ have also been reported by several other investigators [299 - 301]. This augmentation was somewhat unexpected. One possible cause is that it results from a prolongation of the action potential at low $[Na]_o$. Alternatively, it might be

due to an antagonistic action between Na^+ and Ca^{++}, as reported to occur in heart muscle [302]. But the latter possibility is unlikely because in frog's skeletal muscle fibres, an increase of $[Ca]_o$ decreases rather than increases the twitch height as noted by us and several other investigators [303, 304, 226].

To investigate the mechanism for the antagonistic effect of low $[Na]_o$ on the twitch potentiation by opioids, intracellular recording experiments were carried out. These experiments showed that a reduction of $[Na]_o$ from 111.8 mM to 44.7 mM caused a marked reduction in the action potential amplitude and in its maximal rate of rise. This was not unexpected because it is well known that the above electrical parameters of the membrane are linearly proportional to the logarithm of the Na^+ concentration in the external fluid [253, 305].

When methadone (5 x 10^{-5} M) in low-Na^+ Ringer's solution was tested on the frog sartorius muscle, it caused a further depression in the amplitude of the action potential and its maximal rate of rise. Therefore, the addition of the local anesthetic effect of the opioid on to an already impaired Na^+ conductance process in the muscle apparently caused the observed depression of the twitch. This is understandable because a steep $[Na]_o$ vs twitch relation exists in this region of extracellular Na^+ concentrations [306].

The rapid reversal of the depressant effect of the opioid and the manifestation of a potentiated twitch on providing the muscle with the regular amount of Na^+ clearly indicates that the two effects of the drug are exerted simultaneously on the muscle and that under normal conditions the twitch potentiating effect of the opioid is predominant and is therefore exhibited. The depressant effect only becomes evident at high concentrations of the opioid, when the drug is exposed to the muscle for a prolonged period of time or when the extracellular Na^+ concentration is greatly reduced. It is conceivable that the magnitudes of these two effects vary with the different opioids and thereby account for differences in their actions on the twitch. It is also possible that etorphine, the only opioid amongst those tested that did not potentiate the twitch, has a higher depressant component and therefore its overt response is only a twitch depression.

To further delineate the site of opioid action we tested a quaternary derivative of naloxone on the twitch of the frog toe muscle. This compound is identical in structure to naloxone excepting for the presence of an additional methylbromide moiety at its tertiary nitrogen atom. Quaternary narcotic antagonists are effective in antagonizing opioid effects and they often have been employed to delineate the peripheral from the central actions of opioids [307] since these antagonists do not

penetrate the blood brain barrier. These compounds are also believed to be unable to penetrate effectively into the muscle fibre membrane because of their quaternary nature. In our experiments, quaternary naloxone failed to potentiate the twitch even when used in concentrations as high as 10^{-3} M (Fig. 13), whereas naloxone tested on the same preparation exhibited its usual twitch potentiating ability. This implied that opioids do not act on the outer or extracellular surface of the muscle fibre membrane but act presumably by dissolving in the membrane.

The results discussed thus far allow the suggestion that opioids act by facilitating the E-C coupling process in muscle. The precise manner in which they may do so cannot be stated with certainty because the mechanism whereby depolarization of the t-tubular membranes leads to release of Ca^{++} from the sarcoplasmic reticulum is still a matter of intense debate (see 229, 240, 308, 226, 309, 310, 311, 243]. The theories that have been proposed to explain this process may be divided into five types:

- Charged particles in the t-tubular membrane more under the influence of the electric field across the membrane, and this movement leads to the release of Ca^{++} from the sarcoplasmic reticulum [312].
- The t-tubules are electrically coupled to the sarcoplasmic reticulum and depolarization causes a potential change in the sarcoplasmic reticulum, which

leads to Ca⁺⁺ release [313].

- Ca⁺⁺ enter through the *t*-tubular membranes and trigger E-C coupling [314].

- Na⁺ enter through the *t*-tubular membranes and trigger E-C coupling [315, 316].

- Depolarization induces release of membrane bound 'trigger Ca⁺⁺' which causes massive release of additional Ca⁺⁺ from the sarcoplasmic reticulum [220 - 226].

From the various theories listed above, the 'trigger' Ca⁺⁺ hypothesis offers a possible explanation for the opioid effect on the twitch. The antagonistic effect of an elevated extracellular concentration of Ca⁺⁺ on the potentiated twitch raises the possibility that opioids act by rendering the pool of 'trigger' Ca⁺⁺ more labile. If the opioids are causing such an effect then they would be expected to produce a potentiated twitch, because every stimulus would then be followed by the release of a greater amount of the 'trigger' Ca⁺⁺ which would presumably cause a release of a greater amount of 'activator' Ca⁺⁺ from the sarcoplasmic reticulum. According to the 'trigger' Ca⁺⁺ hypothesis, the increased $[Ca]_o$ would increase the binding of the 'trigger' Ca⁺⁺ to the inner membrane of the *t*-tubules and thereby make them less liable to be displaced by the opioid.

It is technically not feasible at present to study directly the proposed enhancement of the release of 'trigger' Ca^{++} by an opioid during an electrical stimulus. We therefore decided to test this possibility indirectly. To achieve this objective we studied the effects of opioids on the maximally summated muscle response evoked by closely spaced multiple electrical stimuli (number of pulses, 2 - 7). Our rationale for this study was that closely spaced repetitive electrical stimulation of the muscle would cause a reduction in the stores of the membrane bound 'trigger' Ca^{++} due to the insufficient time between stimuli to permit a replenishment of these stores. If a drug causes the release of an additional amount of 'trigger' Ca^{++} during stimulation, then its response to a single shock would be potentiated but the percent increase in the potentiation might be expected to decrease with an increase in the number of pulses composing the train of electrical stimuli. Our experiments showed that this was actually the case. An increase in the number of pulses in the pulse train did cause a decrease in the percent increase in the response height, in presence of twitch potentiating concentrations of various opioids (Fig. 15 - 19). Therefore, if our original assumption was true (i.e., there exists a limited pool of 'trigger' Ca^{++} bound to inner surface of the t-tubular membrane), then these results would suggest that opioids enhance the displacement of the 'trigger' Ca^{++} during a

stimulus.

That this decrease is not due to the muscle attaining its maximum contractile ability was indicated by the fact that the magnitude of the tetanic response in this muscle is about 3 - 5 times that of the single twitch and that the former amplitude was never attained by the summated responses. These experiments do support our original contention that opioids potentiate the twitch in the frog skeletal muscle by increasing the release of 'trigger' Ca^{++} during an electrical shock.

There is however, an alternative explanation for the phenomenon just described. The decrease in the percent increase in the magnitude of the summated responses, in presence of the opioid might be a consequence of the local anesthetic property of the opioid exhibiting frequency dependency and use dependency. Local anesthetics are known to exhibit such properties because the drug molecule gains access to the receptor only when the 'gates' at the inner face of the Na^+ channel are open and because the affinity of the local anesthetic for the receptor in the Na^+ channel is voltage dependent [317]. If such a property is shared by opioids then it might explain the decrease in the percent increase in the response height following stimulation of the muscle with trains of 2-7 closely spaced electrical pulses. At this point in time we do not have any firm experimental evidence to support this possibility and therefore we favour

our earlier hypothesis that opioids act by effecting the release of additional 'trigger' Ca^{++} during an electrical stimulus.

Our observation that opioids do not potentiate the tetanic response but cause it to decline in an irregular fashion (during the stimulation) prompted us to carry out a few preliminary experiments to examine this phenomenon. It was observed that the maximal tetanic response was unaffected by a reduction of $[Ca]_o$ from 1.08 to 0.27 mM (a 75% reduction) although the twitch was potentiated under similar conditions (Fig. 49). The tetanic response was, however, much more sensitive to a reduction in $[Na]_o$ than was the twitch (Fig. 51). A comparison of the effects of reductions in the extracellular concentrations of the two ions, (Na^+ or Ca^{++}), with the effects of the opioids on the tetanic response suggests that the opioids act by causing a decreased influx of Na^+ during the response. This again could be a consequence of the possible frequency dependency and use dependency property of the opioids, as discussed earlier. That local anesthetics do produce such an effect was shown by the rapid suppression of the tetanic response by procaine (1 mM) whilst causing only a small depression of the twitch response (Fig. 52).

6. SUMMARY AND CONCLUSIONS

1. The effects of several opioids (morphine, meperidine, methadone, codeine, fentanyl, etorphine, dextromethorphan, dextrorphan, levorphanol, naloxone, quaternary naloxone, Mr 2096, ketazocine, ethyl ketazocine, met-enkephalin and leu-enkephalin) were examined on the twitch responses of the isolated, curarized, and electrically stimulated frog toe muscles. All the opioids tested, excepting etorphine and quaternary naloxone, produced a dual action on the twitch response; potentiating action at low drug concentrations and a potentiation followed by an inhibitory action at higher concentrations.

2. The twitch potentiation was found to be nonstereospecific and resistant to antagonism by all the opioid antagonists tested, viz., naloxone, naltrexone, Mr 2096, or phenoxybenzamine. This implied that the opioid-effects are not mediated by any one of the well known and well documented subtypes of opioid receptors but are due to the activation of nonstereospecific and naloxone-resistant opioid receptors which have been postulated to exist elsewhere by several investigators.

3. The inhibitory action also was naloxone-resistant and

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is probably due to a nonspecific local anesthetic effect of the opioids on the electric properties of the frog skeletal muscle fibre membrane.

4. The opioid-induced twitch potentiations were not caused by recruitment of additional muscle fibres or by a repetitive discharge in individual muscle fibres.

5. A study of the effects of methadone on muscle responses to closely spaced electrical pulses indicated that it decreased the critical fusion frequency of the muscle. The drug also increased the time required by the twitch response to attain its peak amplitude. These changes suggest that opioids prolong the duration of the active state of the muscle.

6. The twitch potentiating effects of opioids were rapid in onset and were rapidly reversed following washout of the drugs. The rapid kinetics of build-up of the full potentiating effects of the opioids suggest that they act directly on the membrane of the muscle fibre. The rate of penetration of opioids into the myoplasm is known to be too slow to account for the rapid development of the muscle's potentiation, and thus internally located sites were eliminated as the point of action of the opioids. These conclusions were corroborated by corresponding considerations of the rapid reversal that occurred when the muscle that had been maximally potentiated by an opioid was restored to

drug-free Ringer's solution.

7. Opioids do not promote the influx of Ca^{++} through the slow Ca^{++} channels during depolarization of the muscle fibre membrane. This was indicated by the lack of any potentiating effect of methadone on K^+-induced contractures. The above conclusion was reinforced by the finding that D-600, a Ca^{++} channel blocker, did not affect the twitch potentiation produced by opioids, although it effectively antagonized the contractures.

8. The twitch potentiating effects of opioids were antagonized by raising the extracellular concentration of Ca^{++} from 1.08 mM to 8.64 mM or above.

9. Opioids do not potentiate twitches by displacing the superficially bound membrane Ca^{++} from the muscles. Morphine or meperidine, when used in concentrations that produced good twitch potentiations, did not displace any ^{45}Ca from that pool of Ca^{++}. The significance of the slight displacement of ^{45}Ca observed with methadone and its involvement in this opioid's effects on the twitch is not clear.

10. Low, twitch-potentiating concentrations of opioids did not produce any change in the electrical properties of the muscle fibre membrane within 5 min of drug exposure. Higher concentrations, however, did prolong the action potential duration. A contribution of this effect to the observed twitch potentiation cannot be

ruled out.

11. An increase in the extracellular concentration of Ca^{++} did not antagonize the opioid-effects on the electrical properties of the muscle fibre,as it antagonized the drug-effects on the twitch. This implied that no causal relationship exists between the changes in the electrical properties of the membrane produced by the opioids and their twitch potentiating abilities.

12. High concentrations of compounds known to increase the myoplasmic levels of Ca^{++} (viz., caffeine or A23187) antagonized the opioid effects on the twitch. Since these compounds are also known to produce permanent structural changes in the muscles in the concentrations used, the significance of the observed antagonism is not known.

13. The twitch potentiating effects of opioids were antagonized by decreasing the concentration of extracellular Na^+. This antagonistic effect, however, is most likely due to the local anesthetic effect of the opioids acting in concert with the reduced extracellular concentration of Na^+ to produce a depression of the twitch response.

14. Quaternary naloxone did not potentiate the twitch whereas naloxone tested on the same preparations exhibited its usual twitch potentiating abilities. This implied that opioids do not act on the outer or

extracellular surface of the muscle fibre membrane but act presumably by dissolving in the membrane.

15. A study of the effects of opioids on the maximally summated muscle responses to closely spaced multiple electrical pulses (number of pulses, 2-7) revealed that an increase in the number of pulses in the pulse train causes a decrease in the percent increase in the response height, in presence of twitch potentiating concentrations of opioids. It is proposed that this effect is due to a depletion of the stores of 'trigger' Ca^{++} following stimulation with trains of closely spaced multiple pulses.

16. The results suggest that opioids produce their effects on the twitch by facilitating the process of excitation-concentration coupling in the muscle. It is proposed that they do this by causing the release of an additional amount of 'trigger' Ca^{++} during the electrical stimulus.

17. The plateau of the tetanic response fell irregularly in the presence of twitch-potentiating concentrations of opioids. Preliminary experiments suggest that this is due to a local anesthetic effect of the opioids.

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