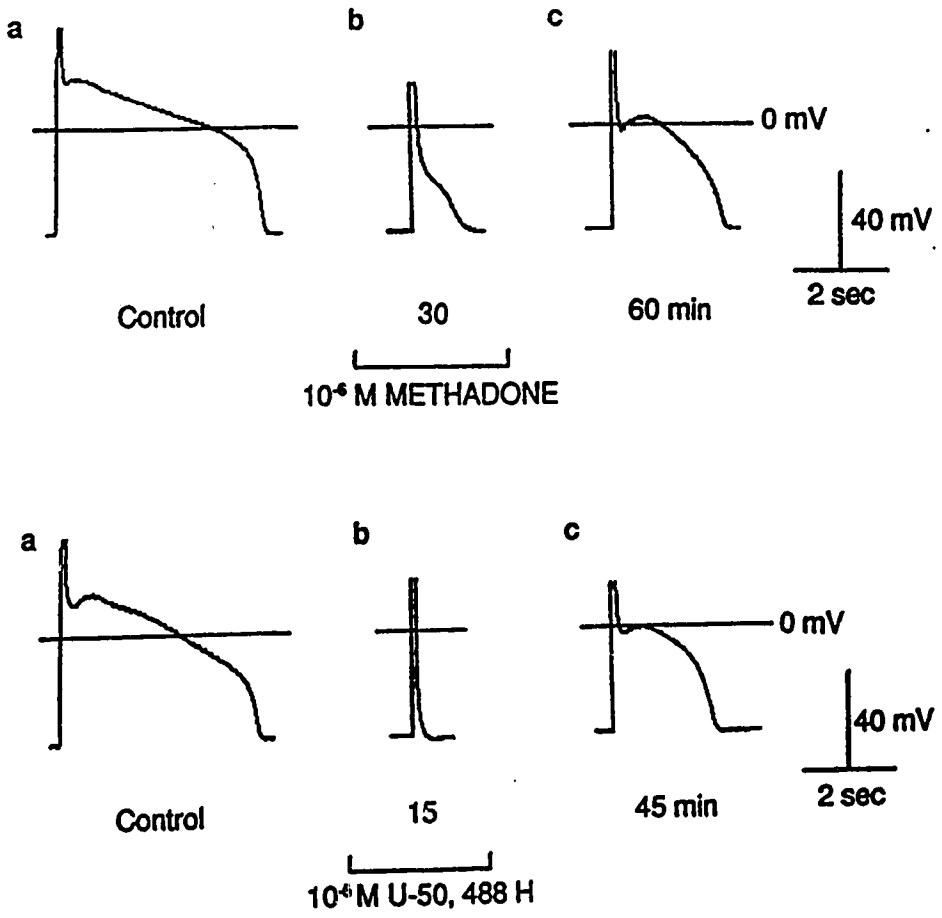

FIG. 15 Effects of methadone (10^{-6} M) and U-50,488H (10^{-5} M) on Ca^{++}-dependent slow APs in frog sartorius muscles. Slow APs were recorded every 15 min. (a) Control response; (b) test during exposure to a drug; (c) recovery response after keeping the muscle for 30 min in a drug-free solution. The upper horizontal line in each panel give the zero potential level. The tops of the stimulus artifacts have been removed.

similar way, Ca^{++}-dependent slow APs obtained by using 3 mM $[Ca]_o$ were inhibited by several other non-peptide opioid agonists and the antagonist, naloxone in a concentration-dependent manner (Table 8).

All the opioids tested produced essentially similar qualitative changes. Thus, it took usually 15 to a maximum of 45 min (in few cases) to develop the maximum effect of the opioids on the Ca^{++}-dependent slow APs, irrespective of the concentration of the opioid applied. Usually, a recovery to 30-70% of control was observed in 1 hr following drug removal. All the non-peptide opioids tested produced a more dramatic decrease in the duration of the slow APs than in the amplitude of the slow APs. In few experiments, tetraethylammonium (TEA) (40 mM) was added to the experimental solution to block K^+ conductance. Meperidine (10^{-5} M) and Methadone (10^{-6} M and 10^{-7} M) caused 82.1, 84.2 and 66.6% inhibition of the area of Ca^{++}-dependent slow APs, respectively (n=3, each) even in the presence of TEA.

4.6.3 Effect of opioid peptides on Ca^{++}-dependent slow APs

Although the non-peptide opioids mentioned above were quite effective in reducing or blocking these APs in frog skeletal muscle, various opioid peptides showed little or no effect on these slow APs (Table 9). Thus, dynorphin (10^{-5} M) caused only a 8.8% inhibition of the area of Ca^{++}-dependent slow APs when tested for 150 min (Fig. 16). Leucine-enkephalin in a concentration as high as 10^{-4} M produced only a 17.9% inhibition of the area of these APs.

TABLE 8

Inhibition of Ca^{++}-dependent slow APs by various non-peptide opioids in frog skeletal muscle

Drug	Maximum Inhibition of Area* (%)			
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Morphine	--	88.7 ± 5.1 (3)	--	--
Meperidine	--	83.5 ± 2.3 (3)	--	--
Methadone	--	98.3 ± 1.7 (3)	80.8 ± 5.5 (3)	68.0 ± 1.0 (2)
Levorphanol	98.3 ± 1.7 (3)	73.7 ± 6.5 (3)	55.8 ± 1.9 (3)	--
Dextrorphan	97.3 ± 1.8 (3)	70.5 ± 3.9 (3)	57.4 ± 1.3 (3)	--
Naloxone	75.0 ± 4.6 (3)	44.6 ± 5.5 (3)	9.1 ± 3.2 (3)	--
U-50,488H	--	100.0 ± 0.0 (3)	81.2 ± 5.0 (3)	43.2 ± 8.3 (3)

NOTE: Mean ± S.E.M.; -- , not tested. Number in bracket = number of experiments.
* Area under the slow AP, eliminating the stimulus artifact, was measured using the Turbo Pascal program.

TABLE 9

Lack of effect of opioid peptides on the Ca^{++}-dependent slow APs in frog skeletal muscle

Drug	Maximum Inhibition of Area* (%)		
	10^{-4} M	10^{-5} M	10^{-8} M
Leu-enkephalin	17.9 ± 4.2 (3)	---	---
Morphiceptin	---	10.7 ± 4.2 (3)	---
Dynorphin	---	8.8 ± 3.8 (3)	3.7 ± 1.8 (3)

NOTE: Means ± S.E.M., ---, not tested.
Number in bracket = number of experiments.
* Area under the slow AP, eliminating the stimulus artifact, was measured using the Turbo Pascal program.

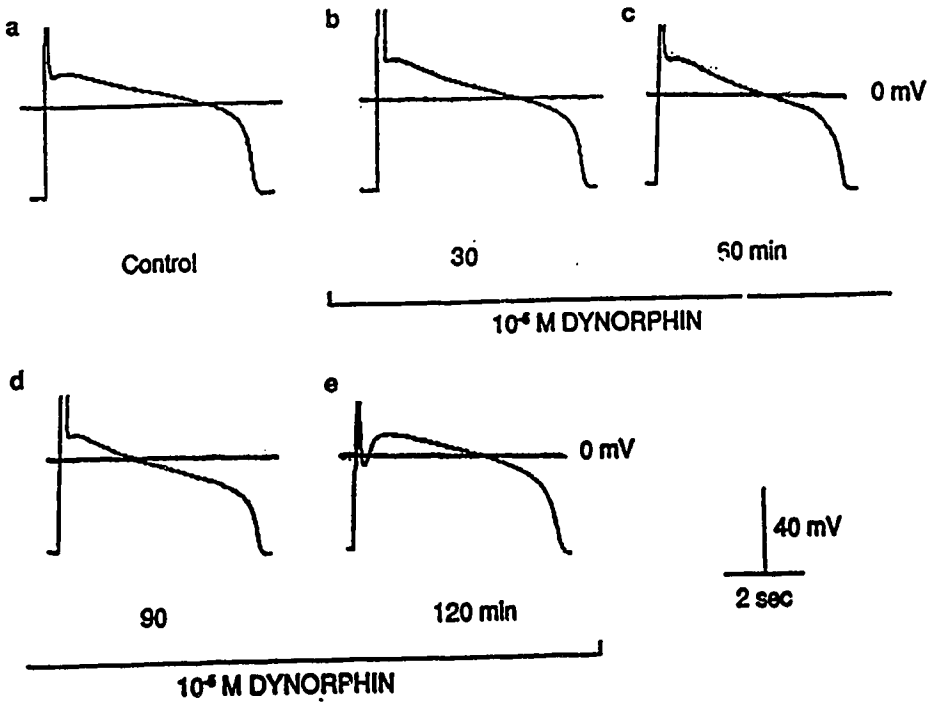

FIG. 16 Lack of effect of dynorphin (10^{-5} M), an opioid peptide, on Ca^{++}-dependent slow APs produced in frog sartorius muscle. Slow APs were obtained using a Na^{+}-free, Cl^{-}-free, high-K^{+} medium and recorded every 15 min. Horizontal line under the records indicates exposure to dynorphin (10^{-5} M). Times below the records indicate the time after the start of exposure to dynorphin. (a) control response; (b) to (e) tests during exposure to dynorphin. The upper horizontal line in each panel give the zero potential level. A prominent stimulus artifact is visible at the start of each slow AP.

4.7 INVESTIGATION OF THE POSSIBLE INVOLVEMENT OF A STEREOSPECIFIC OPIOID RECEPTOR IN THE DEPRESSION OF Ca^{++}-DEPENDENT SLOW APs PRODUCED BY OPIOIDS

4.7.1 Studies with opioid stereoisomers

Levorphanol, a potent opioid analgesic, and dextrorphan, its corresponding inactive isomer, exhibited no significant differences in their ability to produce inhibition of Ca^{++}-dependent slow APs (Fig. 17). This result clearly demonstrated that the effective opioids produced a nonstereospecific blockade of the voltage-dependent slow calcium channels in frog skeletal muscle.

4.7.2 Studies with opioid antagonists

To test this point further, naloxone was used as an opioid antagonist. As with K^+ contractures, when naloxone was tested alone in high concentrations, it produced inhibition of slow APs (Table 8). Since lower concentrations of naloxone (10^{-6} M and lower) showed little effect on Ca^{++}-dependent slow APs, these concentrations were used to test for an antagonistic effect. However, no antagonism was observed with naloxone in concentrations ranging from 10^{-10} M to 10^{-6} M (Table 10). In fact, there seemed to be additive effects.

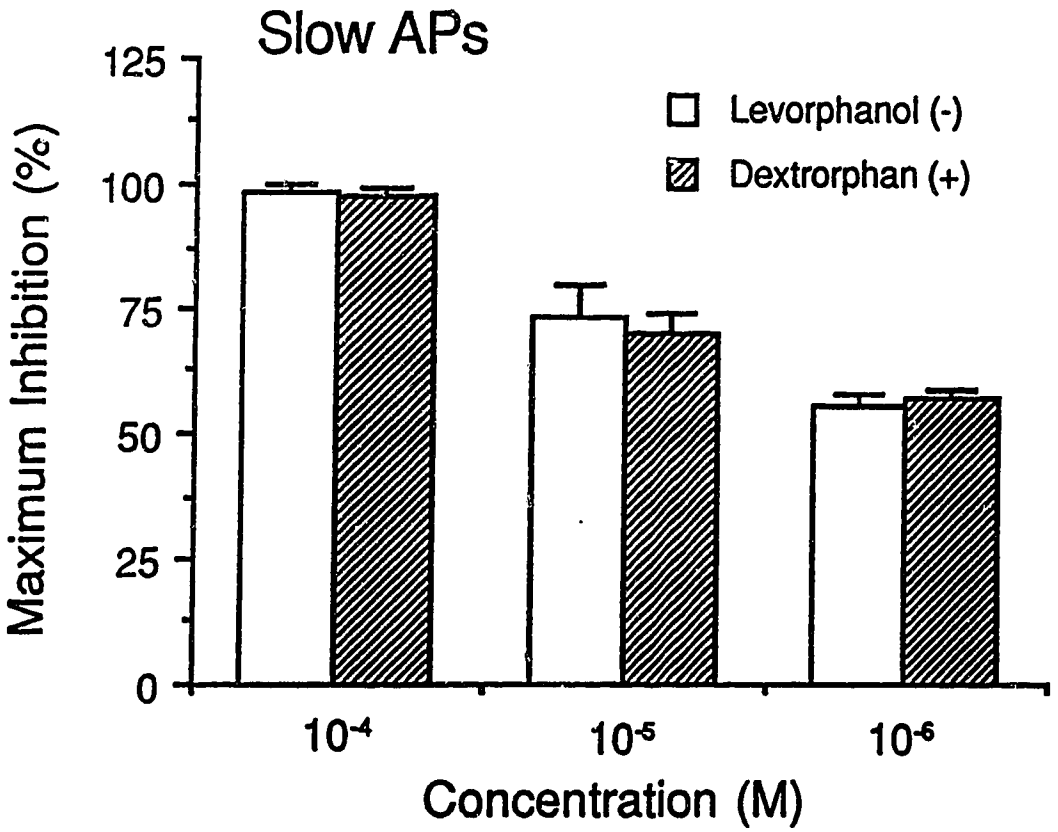

FIG. 17 Maximum inhibition (%) of the area of Ca^{++}-dependent slow APs produced by different concentrations of stereoisomers; levorphanol, analgesically active isomer, and dextrorphan, an inactive isomer, in frog skeletal muscle. Means ± SEM (n=3, each).

TABLE 10

Lack of antagonism of naloxone and norbinaltorphimine on the slow AP inhibition produced by opioid agonists in the isolated sartorius muscle of frog

Agonist (concen)	+	Antagonist (concen)	Maximum inhibition of area (%)	Difference from control value* (%)
Methadone (10^{-6} M)	+	Naloxone (10^{-6} M)	91.0 ± 1.5 (3)	+ 10.2
Methadone (10^{-6} M)	+	Naloxone (10^{-8} M)	86.8 ± 7.7 (3)	+ 6.0
Dextrorphan (10^{-5} M)	+	Naloxone (10^{-10} M)	82.7 ± 4.9 (3)	+ 12.2
Dextrorphan (10^{-5} M)	+	Naloxone (10^{-9} M)	73.3 ± 6.1 (3)	+ 2.8
U-50,488H (10^{-6} M)	+	Naloxone (10^{-7} M)	84.4 ± 4.8 (3)	+ 3.2
U-50,488H (10^{-6} M)	+	Naloxone (10^{-9} M)	84.5 ± 6.0 (3)	+ 3.3
U-50,488H (10^{-6} M)	+	Norbinaltorphimine (10^{-6} M)	83.4 ± 3.1 (3)	+ 2.2
U-50,488H (10^{-6} M)	+	Norbinaltorphimine (10^{-8} M)	85.8 ± 3.1 (3)	+ 4.6

NOTE: Mean ± S.E.M.
Number in bracket = Number of experiments
* See Table 8 for control values (i.e., % inhibition in absence of antagonist)

Naloxone did not antagonize the slow AP inhibition produced by U-50,488H, an opioid agonist specific for the K-receptor subtype (Table 10). Also, norbinaltorphimine, an opioid antagonist specific for the K-receptor subtype showed no antagonism of the inhibition of the slow APs produced by U-50,488H (Table 10). Thus, both naloxone and norbinaltorphimine did not produce any antagonism of the U-50,488H induced depression of Ca^{++}-dependent slow APs.

The results presented above were used to establish a relationship between the inhibitory effects of opioids on K^+ contractures (Table 3 and 4) and Ca^{++}-dependent slow APs (Table 8 and 9). A linear and highly significant correlation (r=0.96) appears to exist between K^+ contracture and Ca^{++}-dependent slow AP inhibition by opioid drugs (Fig. 18). Thus, in general, same rank order of activity was observed despite quantitative differences in apparent potency.

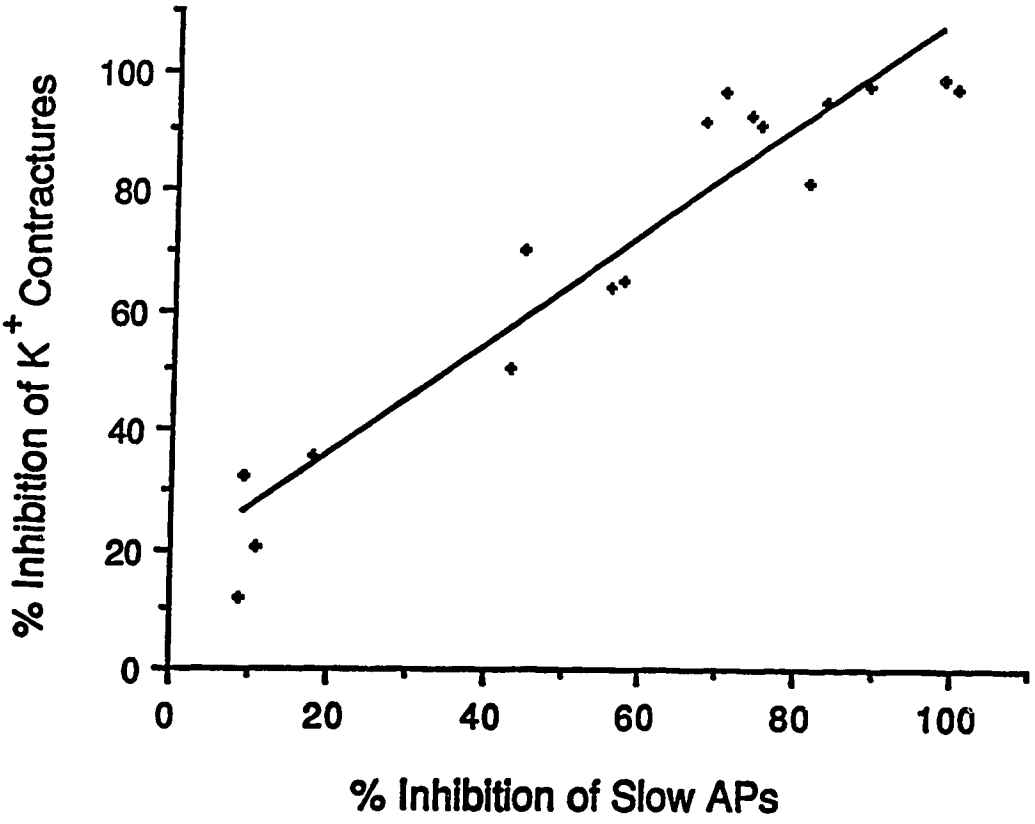

FIG. 18 Correlation between the inhibitory effects of opioids on Ca^{++}-dependent slow APs and K^+ contractures. The plot has a slope of 0.91 and a correlation coefficient r=0.96.

4.8 EFFECT OF FREQUENCY OF K^+ CONTRACTURE TESTS ON THE BLOCK PRODUCED BY NON-PEPTIDE OPIOIDS

As shown earlier, several non-peptide opioids inhibited K^+ contractures (123 mM) in a concentration-dependent manner (Table 3). Little or no reduction of the K^+ contracture was observed in the first test with the high-K^+ solution following the drug application (Fig. 3). However, when tests were repeated at 15 min intervals, block of K^+ contractures was eventually produced (Fig. 3). Thus, it took about 60 min for the complete block to occur with methadone (10^{-5} M), when K^+ contractures were tested every 15 min. This blockade of K^+ contractures by non-peptide opioids was partially reduced when the muscles were kept in the drug solution without testing with the high-K^+ solution for 30 min or more. Thus, methadone (10^{-5} M) completely blocked K^+ contractures in 60 to 90 min when tested with the high-K^+ solution every 15 min. This block was partially recovered (33.5% recovery) by testing the K^+ contracture after a 30 min rest (n=3).

A more complete picture of the effects of methadone (10^{-5} M) on K^+ contractures is presented in Fig. 19. In the experiment shown (Fig. 19), one muscle (filled circles) was tested with the high-K^+ solution every 15 min in methadone (10^{-5} M) and the other muscle (open circles) from the same frog was only tested after 90 min in methadone (10^{-5} M). While in the first toe muscle (filled circles), K^+ contractures were blocked by methadone (10^{-5} M) in 75 min, little or no decrease in

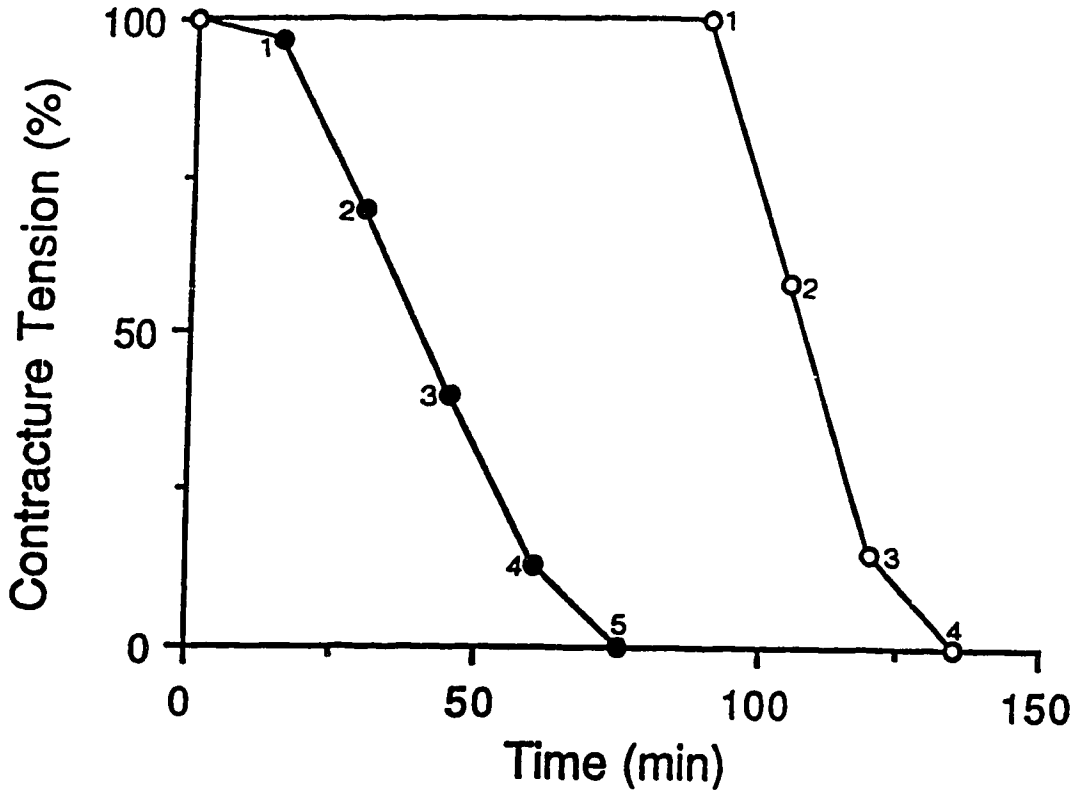

FIG. 19 Effect of timing of K^+ contracture tests on the inhibition produced by methadone (10^{-5} M) in a pair of toe muscles isolated from a single frog. Muscle put in a drug containing solution at time zero. One muscle was tested with high-K^+ solution every 15 min (filled circles, 1-5) and complete inhibition of high-K^+ contracture was observed by the fifth test with the high-K^+ solution. Another muscle from the same frog was tested with high-K^+ solution after a 90 min exposure to methadone (10^{-5} M) (open circles, 1) and no inhibition of high-K^+ contracture was observed. Treating the same muscle further every 15 min produced complete block of K^+ contractures by the fourth test with the high-K^+ solution (open circles, 4).

the initial response of the high-K^+ solution was obtained with the second toe muscle (open circles) after a 90 min exposure to methadone (10^{-5} M) (Fig. 19, n=3). Subsequently, testing the second toe muscle (open circles) every 15 min eventually blocked K^+ contractures (Fig. 19). Similar results were obtained with U-50,488H (10^{-5} M), *i.e.*, a complete block of the K^+ contractures in 60 to 75 min when the K^+ contractures were tested every 15 min and little or no effect on the first K^+ contracture even after a long (90 to 120 min) exposure to U-50,488H (10^{-5} M) (n=3).

Using methadone (10^{-5} M) as a prototype, a more detailed study was conducted of the effect of changing the frequency of K^+ contracture tests; as shown in Fig. 20, the inhibition could be increased (or accelerated) by increasing the frequency of the K^+ contracture tests. Thus, when these contractures were evoked every 10 min, complete block was obtained by 40 min, while it took about 75 min for the complete block to occur when contractures were evoked every 15 min (n=3, each). With high-K^+ tests every 30 min, the same concentration of methadone produced only a 48% inhibition, while this block completely disappeared, if high-K^+ tests were made every 60 min (n=2, each) (Figs. 20 and 21). Thus, blockade of K^+ contractures by non-peptide opioids was found to be dependent on the frequency of K^+ contracture tests.

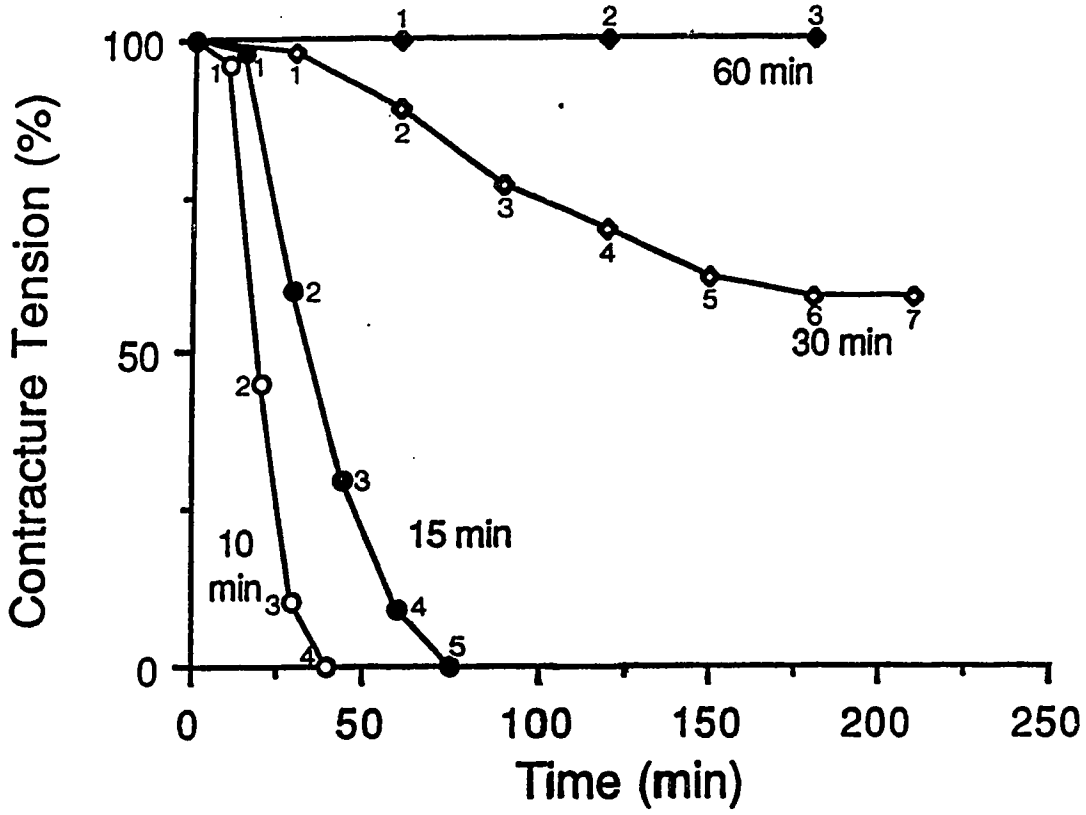

FIG. 20 Inhibition of K^+ contractures produced by methadone (10^{-5} M), dependent on the frequency of K^+ contractures produced in frog toe muscle. Frequency of K^+ contractures: every 10 min (0-0, 1-4), every 15 min (●-●,1-5), every 30 min (◇-◇, 1-7) and every 60 min (◆-◆, 1-3). Numbers indicate the application of high-K^+ solution with different intervals. For details see the text.

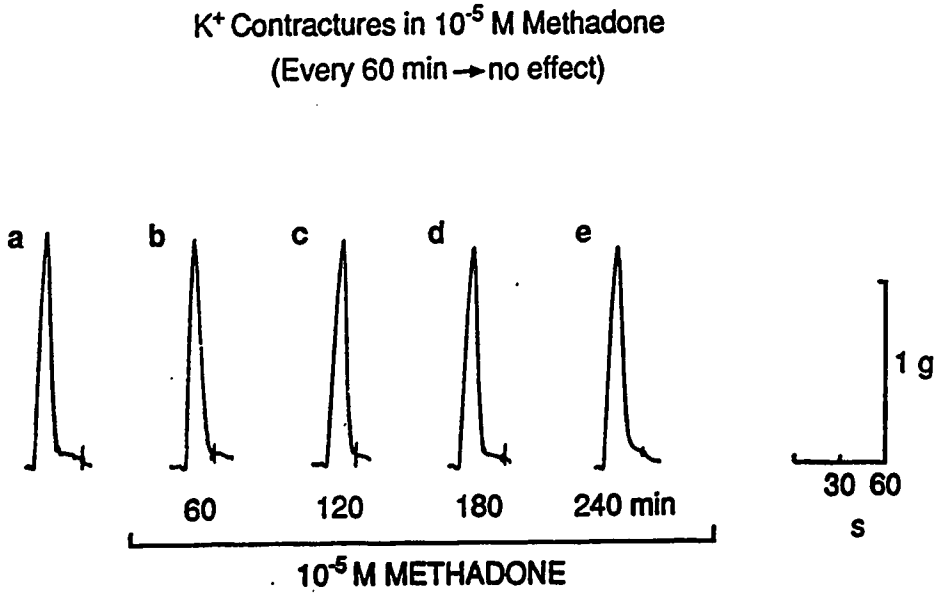

FIG. 21 Lack of effect of methadone (10^{-5} M) when K^+ contractures (123 mM) were induced every 60 min. Horizontal line under the records indicates exposure to methadone (10^{-5} M) in choline-Ringer's solution. Times below records indicates the time after exposure to methadone. (a) Control response; (b - e) tests during exposure to methadone.

4.9 EFFECT OF A Cl^--FREE, HIGH-K^+ SOLUTION ON THE K^+ CONTRACTURE INHIBITION PRODUCED BY NON-PEPTIDE OPIOIDS

The inhibitory effect of the non-peptide opioids on K^+ contractures developed slowly. Even with higher concentrations (10^{-5} M and more), it took a minimum of 60 min for the complete block of K^+ contractures by non-peptide opioids (see, section 4.1.2). In contrast, the blockade of the Ca^{++}-dependent slow APs by the non-peptide opioids was produced in about 15 min, irrespective of the concentration of the drug employed (see, section 4.6.2). To resolve this discrepancy, a Cl^--free, high-K^+ (10 mM) solution was employed. This experimental solution was similar to the solution used for generating Ca^{++}-dependent slow APs. The effect of this particular experimental solution on the non-peptide opioid-induced block of the K^+ contractures was studied.

In a Cl^--free, Na^+-Ringer's solution with normal $[K]_o$ (2.47 mM, RMP= -90 mV), it took an average 75 min (n=3) for the complete block of the K^+ contactures with methadone (10^{-5} M), which is the same time as is required for blocking K^+ contractures using a choline-Ringer's solution. Increasing $[K]_o$ in a Cl^--free solution to 5 mM (RMP=-75 mV) reduced the time to an average of 60 min for the complete block of the K^+ contractures (n=3). A further increase in $[K]_o$ to 10 mM (RMP=-60 mV), resulted in a complete block of the K^+ contractures by the first test (15 min) with high-K^+ solution in methadone (10^{-5} M) (Fig. 22, n=3).

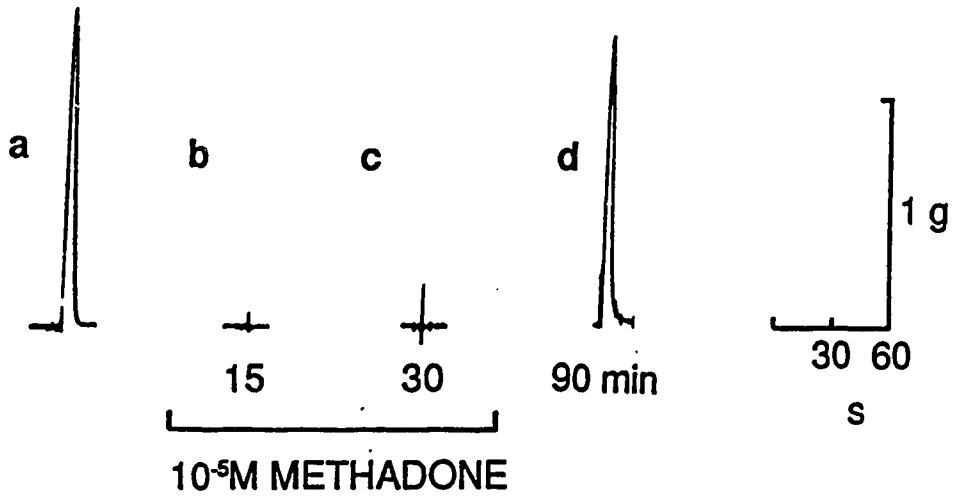

FIG. 22 Blockade of K^+ contracture produced by the first test (15 min) with high-K^+ solution by methadone (10^{-5} M) in a Cl^--free, high-K^+ (10 mM) medium. Times below the records indicates the time after exposure to methadone (10^{-5} M), (a) control response; (b) and (c) tests during exposure to methadone; (d) recovery response after 60 min.

Similarly, U-50,488H (10^{-5} M) completely blocked the K^+ contractures by the first test (15 min) in a Cl^--free, high-K^+ (10 mM) solution (n=3). In separate experiments, U-50,488H (10^{-7} M) produced a 98% inhibition of the K^+ contractures in a Cl^--free, high-K^+ solution, whereas in normal Ringer's solution this concentration produced only a 45% inhibition of the K^+ contractures (n=3, each).

4.10 MISCELLANEOUS PRELIMINARY EXPERIMENTS

4.10.1 Effect of Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) on K^+ contractures and Ca^{++}-dependent slow APs

DTALE has been reported to have calcium-agonist activity in a vascular smooth muscle preparation [193]. This calcium-agonist effect of DTALE was found to be naloxone-insensitive, but it could be reversed by the calcium-channel antagonists verapamil and nitrendipine [193]. Thus, it was of interest to study the effect of DTALE on a skeletal muscle preparation.

The results obtained in one of the experiments with DTALE (10^{-5} M) are shown in Fig. 23. In this experiment, DTALE (10^{-5} M) did not produce any effect on submaximal K^+ contractures (25 mM). Similarly, 10^{-4} M (n=3) and 10^{-5} M (n=3) DTALE had no effect on 25 and 40 mM K^+-induced contractures, when tested for 150 min. In other experiments, DTALE (10^{-5} M, n=3) produced no effect on Ca^{++}-dependent slow APs.

4.10.2 Effect on haloperidol on voltage-dependent slow calcium channels

Recently, haloperidol was reported to bind with high affinity to σ-receptor sites [194,195]. Therefore, it was of interest to study effect of haloperidol on voltage-dependent slow calcium channels in skeletal muscle.

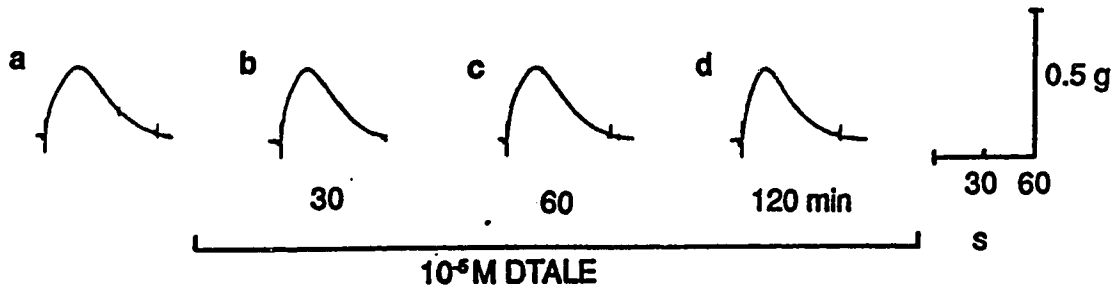

FIG. 23 Lack of effect of Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) on K^+ contractures (25 mM) of frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to DTALE. Times below records indicate the time after the start of the exposure to DTALE. (a) Control response; (b - d) tests during exposure to DTALE.

Haloperidol in concentrations of 10^{-5} M and 10^{-6} M produced a 100% (n=3, Fig. 24) and a 56% (n=3) inhibition of K^+ contractures, respectively. Similarly, haloperidol (10^{-5} M, n=3) completely blocked Ca^{++}-dependent slow APs in frog skeletal muscle. In a separate experiement, haloperidol (10^{-5} M) also caused a 70% inhibition of caffeine contractures (n=1). In the same concentrations (10^{-5} M and 10^{-6} M), it also produced a 93% (n=3, Fig. 25) and a 65% (n=3) inhibition of twitches in isolated frog toe muscles. For comparison, effects of methadone on the twitch responses were studied. By contrast to haloperidol, methadone produced a marked twitch potentiating effect. Thus, methadone (10^{-4} M and 10^{-5} M) caused a 175% (n=3, Fig. 25) and a 140% (n=3) potentiation of twitch responses, respectively.

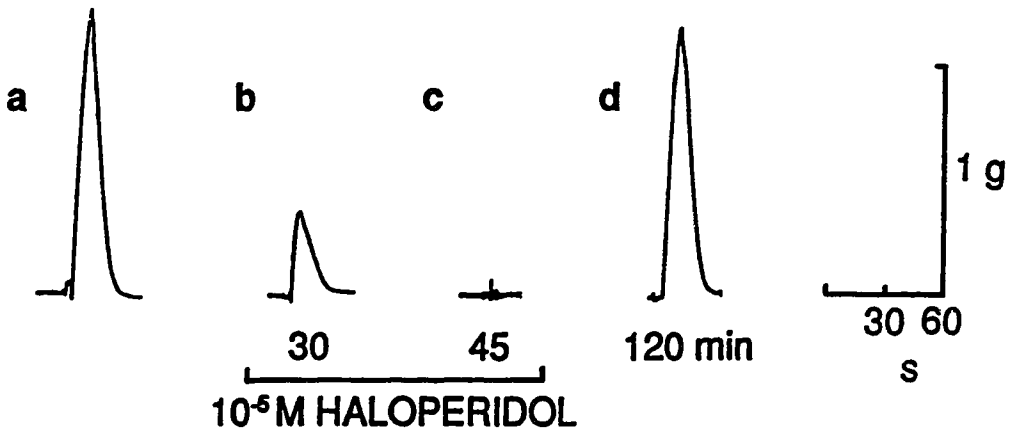

FIG. 24 Effect of haloperidol (10^{-5} M), a σ-receptor agonist, on K^+ contractures (123 mM) of frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to haloperidol. Times below records indicate the time after the start of the exposure to haloperidol. (a) Control response; (b - c) tests during exposure to haloperidol; (d) recovery response.

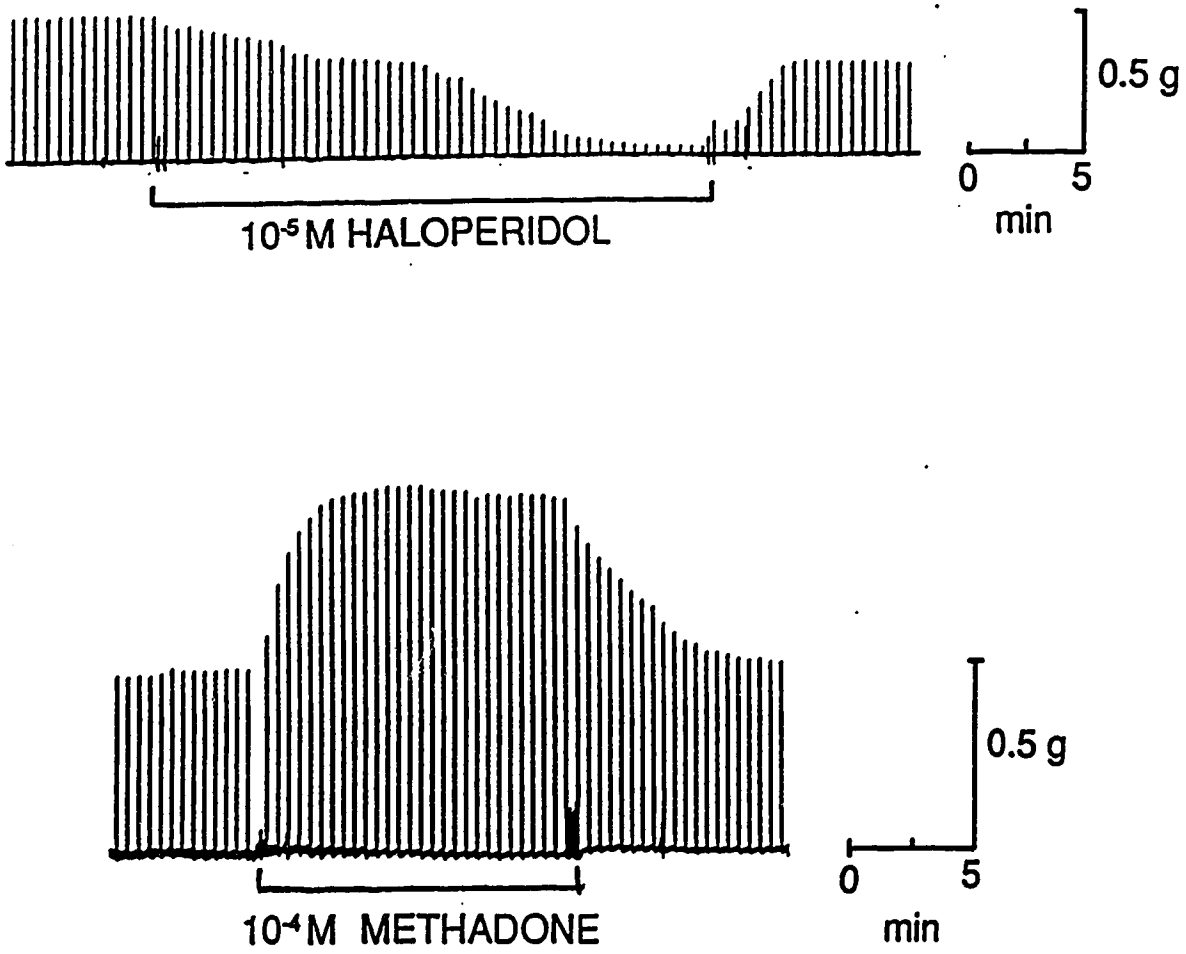

FIG. 25 Twitch inhibition and potentiation in the isolated frog's toe muscle produced by haloperidol (10^{-5} M) and methadone (10^{-4} M), respectively. The preparation was directly stimulated supramaximally once every 30 sec. Horizontal line under the records indicates exposure to a drug.

5. DISCUSSION

5.1 Inhibition of K^+ contractures and Ca^{++}-dependent slow APs by non-peptide opioid drugs

During a study of the ability of many opioid drugs to potentiate twitches in skeletal muscle, a nonstereospecific effect, it was observed that methadone blocked K^+ contractures [180]. To determine whether the inhibition of K^+ contractures caused by methadone was a property unique to that compound, the effects of several opioids with diverse chemical structures were tested on K^+ contractures in frog skeletal muscle. The results of this study were that all of the non-peptide opioids tested produced an inhibition of K^+ contracture similar to that produced by methadone (Table 3). Thus, inhibition of K^+ contractures was observed with different non-peptide opioids, each believed to interact with a particular opioid receptor subtype; e.g., morphine, meperidine and methadone - μ-receptor agonists; U-50,488H - K-receptor agonist; and naloxone - a non-selective opioid antagonist, all produced this effect (Table 3).

The ability of the opioid antagonist naloxone to produce inhibition of K^+ contractures was not surprising because this compound has been demonstrated to be a 'partial agonist' in frog skeletal muscle [196]. Therefore, if used in high concentrations, it showed agonist-like effects. Such effects were also shown by earlier studies of Frank [68] and Frazier *et al.* [197], wherein they observed that the effects of opioid antagonists in high concentrations add on to the effects of opioid agonists.

Surprisingly, while the various non-peptide opioids tested were quite effective in reducing K^+ contractures in low concentrations, various opioid peptides, e.g., leucine-enkephalin - a δ-receptor agonist; morphiceptin - a μ-receptor agonist; and dynorphin - a K-receptor agonist, all had little or no effect on these contractures (Table 4). Even the enzymatically non-degradable peptide analogue D-Ala-leucine-enkephalinamide, had little effect on K^+ contractures (this point will be discussed further in section 5.3 below).

Attempts to associate the non-peptide opioid-induced inhibition of K^+ contractures with one of the proposed opioid receptor subtypes met with failure. The non-peptide opioid-induced inhibition of K^+ contractures in the frog toe muscle was resistant to antagonism by naloxone. The only noticeable effect of naloxone was an increase in the degree of contracture inhibition (Table 5 and 6). Norbinaltorphimine, an opioid antagonist specific for the K-receptor subtype did not antagonize the inhibition of K^+ contractures produced by U-50,488H, a non-peptide opioid agonist specific for the K-receptor subtype (Table 6, Fig. 7).

Analgesic activity, like most of the actions of the opioid drugs, is highly stereospecific, with almost all the activity residing in levo-isomers. If the inhibition of K^+ contracture involved a stereospecific receptor, the levo-isomer should have had higher potency than the corresponding dextro-isomer. However, this was not the

case. Thus, we found that the analgesically active isomer, levorphanol, and its corresponding inactive isomer, dextrorphan, were equally effective in causing inhibition of K^+ contractures (Fig. 6).

The effects on the K^+ contractures were produced at low opioid concentrations (Table 3). Despite this, the effect was not produced via any known stereospecific opioid receptor. The data suggested that the effect on the K^+ contracture is a consequence of the activation of a nonstereospecific, naloxone-resistant opioid receptor. Interestingly, similar nonstereospecific, naloxone-resistant effects of opioids have been reported by others in frog skeletal muscle [198], guinea-pig ileum [199] and rat vas deferens [200].

One treatment that could antagonize the K^+ contracture inhibition produced by the non-peptide opioids was an elevation of $[Ca]_o$ from 1.08 mM to 5 mM (Fig. 8). This antagonism of the drug effect by Ca^{++} may be explained by two possibilities: (1) competition between drug and Ca^{++} ions for a binding site on or near the slow channel, and (2) the increased electrochemical driving force for Ca^{++} influx through the fraction of slow channels not blocked by the drug. The latter mechanism probably operates in all cases.

Non-peptide opioids in concentrations used to block K^+ contractures had no effect on the K^+-induced membrane depolarization (Fig. 10). Also, non-peptide

opioids had no effect on caffeine-induced contractures, indicating that these drugs did not inhibit the release of Ca^{++} from sarcoplasmic reticulum stores. Thus, morphine (10^{-5} M), which produced almost a complete block of the K^+ contractures, did not reduce the maximum tension of the caffeine-induced contracture (Fig. 9). Also, these findings suggest that the internal contractile apparatus is not modified by the non-peptide opioids. Moreover, in a previous study [198], the non-peptide opioids tested were shown to produce twitch potentiation at these low concentrations and caused twitch inhibition only after exposing the muscles to higher concentrations. Thus, exposing toe muscles to 10^{-3} M morphine reduced the twitch by only 50% after 45 min. Thus, the block of K^+ contractures found in this study occurred at concentrations at which the twitch is either potentiated (Fig. 25) or unaffected. This supports the conclusion that the internal contractile apparatus is not depressed at opioid concentrations that block the K^+ contractures. As caffeine-induced contracture were not blocked, and K^+-induced membrane depolarizations were not reduced, and twitches were either potentiated or unaffected at the drug concentrations used, the block of the K^+ contractures cannot be a nonspecific, local anesthetic-like effect.

These results indicate strongly that the effective non-peptide opioids are inhibiting K^+ contractures in skeletal muscle by blocking the voltage-dependent slow calcium channels located in the t-tubules. To further support this conclusion, the effects of opioids on Ca^{++}-dependent slow APs were studied, using intracellular recording techniques.

Recently, electrophysiological studies have shown that opioids block voltage-dependent slow calcium channels in several preparations. Thus, activation of δ-receptors by enkephalins has been shown to reduce calcium conductance in neuroblastoma x glioma hybrid cells [63]. While activation of δ- as well as μ-receptors has been shown to cause inhibition of calcium currents in human neuroblastoma cells [64]. All of these effects were antagonized by naloxone.

Ca^{++}-dependent slow APs in frog skeletal muscle were induced by using a Cl^--free (acetate substituted), Na^+-free (sucrose substituted), high-K^+ (20 mM) solution [192]. These slow APs are produced by the influx of Ca^{++} ions through the voltage-dependent slow calcium channels located in the t-tubules (this point has been discussed in detail, in section 4.6.1). These APs were dependent on $[Ca]_o$ (Fig. 11 and Table 7) and they were abolished by the organic calcium channel blockers nifedipine and diltiazem (Fig. 14).

Several non-peptide opioids inhibited Ca^{++}-dependent slow APs (Table 8 and Fig. 15). The inhibition of the Ca^{++}-dependent slow APs by non-peptide opioids was not prevented by adding tetraethylammonium (TEA), which is known to block membrane K^+ conductance. This suggested that the effective opioids were not blocking the slow APs by increasing the K^+ conductance, but by a direct blockade of the voltage-dependent slow calcium channels.

As was the case for the K^+ contractures, various opioid peptides tested showed little or no effect on these Ca^{++}-dependent slow APs (Table 9). Thus, dynorphin, which has been shown to block slow APs in mouse dorsal root ganglion neurones [54], did not show any effect on Ca^{++}-dependent slow APs in frog skeletal muscle in concentrations as high as 10^{-5} M (Fig. 16).

As observed during the K^+ contracture studies, inhibition of the Ca^{++}-dependent slow APs by the non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone (Fig. 17, Table 10). There was an excellent correlation (r=0.96) between the inhibitory effects of opioids on K^+ contractures and Ca^{++}-dependent slow APs, showing that opioids that are potent (non-peptide opioids) in inhibiting slow APs are also potent in inhibiting K^+ contractures (Fig. 18).

Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) has been reported to have calcium-agonist activity in a vascular smooth muscle preparation [12]. This activity is resistant to antagonism by naloxone, but could be inhibited or reversed by organic calcium channel blockers. This report prompted us to study DTALE's effects on the skeletal muscle preparation. However, DTALE had no effect on voltage-dependent slow calcium channels in frog skeletal muscle.

Recently, haloperidol has been reported to bind with high affinity to the σ-receptor subtype [194, 195]. Therefore, it was of interest to study its effect on the

voltage-dependent slow calcium channels in skeletal muscle. Haloperidol (10^{-5} M) did block K^+ contractures (Fig. 24) and Ca^{++}-dependent slow APs, indicating its ability to block slow calcium channels. However, this effect of haloperidol was not specific to calcium channels. It also inhibited twitches (Fig. 25) and caffeine-induced contractures in the same concentration range. Haloperidol has also been shown to block Na^+ channels in the same concentration range [208]. Therefore, the effects of haloperidol were not specific or limited to calcium channels.

5.2 Frequency- and voltage-dependent block of K^+ contractures by non-peptide opioids

Inhibition of the K^+ contractures by the non-peptide opioids was found to be dependent upon the frequency of K^+ contracture tests. This inhibition could be increased and/or accelerated by increasing the frequency of the K^+ contracture tests (Fig. 20). Thus, methadone (10^{-5} M) completely blocked K^+ contractures in 75 min when the contracture tests were made every 15 min, and this block completely disappeared if high-K^+ tests were made every 60 min (Figs. 20 and 21). These results indicated that more frequent depolarizations increased the non-peptide opioid block of K^+ contractures. This frequency- (use-) dependent inhibition of K^+ contractures by the non-peptide opioids indicated a preferential binding of the non-peptide opioids to their receptors in the open- and/or inactivated-state of the calcium channels (this point will be discussed in detail in section 5.3 below).

One of the differences noted in the studies of K^+ contractures and Ca^{++}-dependent slow APs was that the block of the Ca^{++}-dependent slow APs by the non-peptide opioids developed much quicker than the block of the K^+ contractures. It took usually 15 min to a maximum of 45 min (in few cases) for these drugs to develop their maximum effect on Ca^{++}-dependent slow APs. By contrast, even with high non-peptide opioid concentrations, it took 60 to 90 min to produce a maximum reduction of the K^+ contractures. To resolve the discrepancy between the kinetics of the drug effects on K^+ contractures and Ca^{++}-dependent slow APs, we employed an experimental solution similar to the one used for generating slow APs. Testing the muscles in this solution both accelerated and potentiated the non-peptide opioid-induced inhibition of K^+ contractures. Methadone (10^{-5} M) took a minimum of 60 min to completely block the K^+ contracture in normal Ringer's solution, but it blocked by the first test (15 min) with the high-K^+ solution in a Cl^--free, high-K^+ (10 mM) medium (Fig. 22). Thus, the difference in the onset of block of the voltage-dependent slow calcium channels in the two types of experiments can be eliminated when the solutions bathing the muscles are made similar. These results suggest that the binding of the non-peptide opioid to its receptor is voltage-dependent, with depolarization favoring the binding and thereby accelerating (and potentiating) the block. The receptors in this situation, that is, when the membrane is partially depolarized, are more readily available for the drug molecules to bind.

5.3 Possible mechanisms for the inhibition of voltage-dependent slow calcium channels by non-peptide opioids

The results discussed thus far shed some light on the possible mechanism(s) involved in the blockade of voltage-dependent slow calcium channels by non-peptide opioids (Fig. 26).

Involvement of a G protein (and consequently of a second messenger system) in the responses to opioid-receptor activation has been extensively documented (see section 1.2.4). Thus, the opioid inhibition of adenylate cyclase and consequently cAMP formation has been demonstrated in mammalian brain [75, 76] and the neuroblastoma x glioma hybrid cell line [77, 78]. However, the possible involvement of a second messenger system (Fig. 26, 2) or a coupling of opioid receptor to the G protein, which directly regulates the calcium channel (Fig. 26, 1), in the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels can be ruled out for two reasons: (1) Drugs which produce their response through a G protein (and consequently through a second messenger system) bind to their receptors at the outer surface of the cell membrane; that is, they act from the extracellular site. If in the present study, similar receptors were involved, then various opioid peptides should have been effective in blocking voltage-dependent slow calcium channels, since they can bind to their receptors at the outer surface of the cell membrane and there is no necessity to penetrate the membrane. However, opioid peptides were clearly

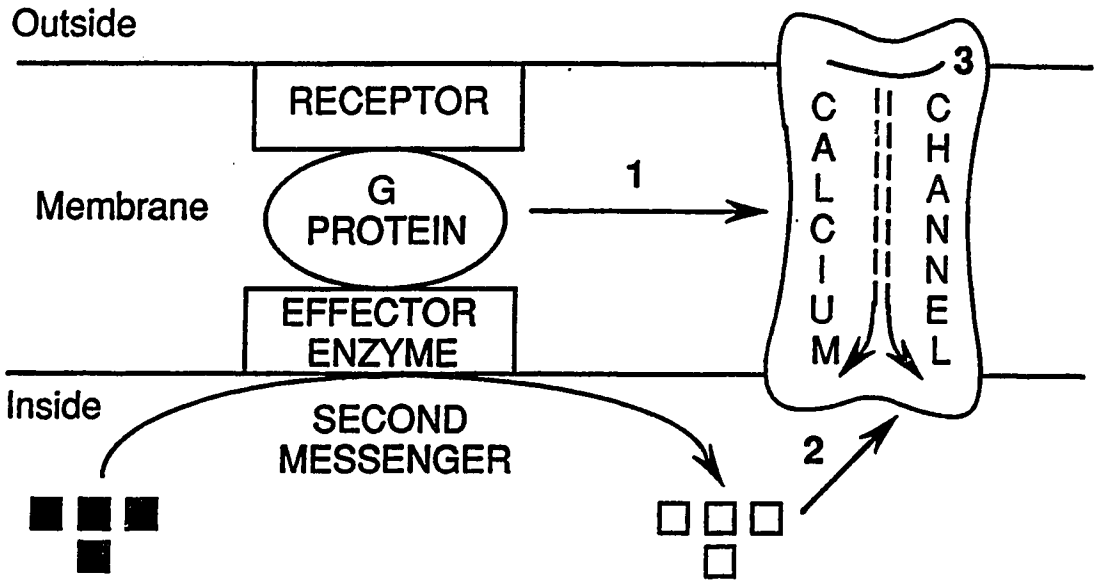

FIG. 26 Schematic representation of possible mechanisms for the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels. (1) Non-peptide opioid receptor coupled to a G protein, which is directly linked to a voltage-dependent slow calcium channel. Thus, activation of opioid receptor will lead to a reduction in calcium current through voltage-dependent slow calcium channels via a G protein that directly regulates the channel activity. (2) Indirect inhibition of slow calcium channels through a 2nd messenger system, e.g., opioid receptor mediated inhibition of adenylate cyclase and consequently cAMP (2nd messenger) formation which will lead to the inhibition of slow calcium channels. (3) Non-peptide opioid binding site inside or near the channel that directly regulates the calcium current through voltage-dependent slow calcium channels.

ineffective in blocking slow calcium channels in skeletal muscle when applied extracellularly. It is possible that the receptor/binding site may be located inside the membrane and since opioid peptides being hydrophilic in nature cannot enter the membrane, and therefore, are ineffective in blocking slow calcium channels. Non-peptide opioids are sufficiently lipophilic to enter/cross the cell membrane, and thus, are effective in blocking the voltage-dependent slow calcium channels in skeletal muscle. However, it is also possible that the receptor is completely different and can be activated only by non-peptide opioids, and not by opioid peptides. (2) In the present study, the non-peptide opioid-induced inhibition of slow calcium channels was found to be frequency- (use-) and voltage-dependent. Such frequency- and voltage-dependent effects are usually not observed with drugs acting through second messenger systems. For example, opioid peptides enkephalin and morphiceptin acting through δ- and μ-receptors, respectively, and involving G proteins in their responses do not show such frequency- or voltage-dependent effects [64].

Although the two reasons presented above make a possible involvement of a G protein (and consequently the second messenger system) seem unlikely, it would be necessary to pursue this possibility further in order to completely rule out the involvement of a second messenger system. In particular, it would be reasonable to test for pertussis toxin-sensitive G protein because most of the responses to opioid-receptor activation studied so far, have shown the involvement of a pertussis toxin-sensitive G protein.

The frequency- and voltage-dependent interaction between the non-peptide opioids and calcium channels in skeletal muscle closely resembles that which occurs between calcium-channel antagonists and calcium channels, and also between local anesthetics and sodium channels [210 - 212]. A simple plugging of the channel by the non-peptide opioids is not consistent with their frequency- and voltage-dependent effects. Our results can be best interpreted within the framework of the modulated receptor hypothesis proposed by Hille [212] to explain the local anesthetic block of sodium channels in nerves and skeletal muscles. In this interpretation, binding of a drug to a receptor located within (Fig. 26, 3) the channel is influenced by the state of the channel which in turn is determined by the membrane potential. This model predicts that ionized drugs can only gain access to the channel-associated receptor via a hydrophilic pathway which is available only when the channels are in the open-state configuration. Uncharged drugs (neutral drugs) can reach the same receptor via this pathway, as well as by a hydrophobic route through the lipid membrane surrounding the channel.

The inhibition of K^+ contractures by non-peptide opioids can be considerably modulated by a change in membrane potential from -90 mV (RMP) to -60 mV. Thus, the inhibition of K^+ contracture by the first test with a high-K^+ solution is observed when the membrane potential is -60 mV and there is no necessity of repeated tests (frequency-) with the high-K^+ solution (Fig. 22). This voltage is too negative to completely open the calcium channels. One interesting possibility that is consistent with the modulated receptor hypothesis is that effective non-peptide

opioids are shifting the steady-state inactivation curve for slow calcium channels towards more negative potentials, that is, in the hyperpolarizing direction. Such a shift in inactivation curve has been often reported with dihydropyridine-type organic calcium-channel blockers [126, 202-204]. Such an effect would suggest that the non-peptide opioids preferentially block channels that are in the inactivated state. However, this possibility remains to be tested.

Another possibility is that the calcium channels are partly but continuously active in partially depolarized muscle. At low levels of K^+ depolarization, there is a sustained influx of calcium in the frog skeletal muscle [205, 206, 207]. Thus, these active calcium channels in partially depolarized muscle might allow the non-peptide opioids to reach their receptors in the channels more effectively than in the normally polarized cells. This would result in quicker block of calcium channels. For this mechanism to occur, drug molecules would have to use a hydrophilic pathway from the cytosolic side of the cell to reach their receptors. The Ca^{++} channel has a selectivity filter of about 6 A° on its extracellular side [214]; this might not allow the non-peptide opioid molecules to enter the channel from the extracellular side, as their molecular size is usually more than 12 A° [216]. So the drugs would have to cross the membrane and enter the channel from cytosolic side in the ionized form. It is interesting to note that most of the non-peptide opioids get extensively ionized at the cytosolic pH and probably enter the channel easily through the hydrophilic pathway. It is also possible that the non-peptide opioid drug molecules may be using

both pathways, that is, hydrophobic pathway through the lipid membrane in their uncharged form and the hydrophilic pathway for the drug molecules, which have crossed the membrane and are ionized in the cytosol. However, more experiments would be necessary to confirm these suggestions. In particular, experiments using the permanently charged (quaternary) non-peptide opioids would be quite useful in testing above possibilities.

5.4 Future recommendations to pursue this project

The voltage-dependent slow calcium channel is a target for a number of drugs collectively called the organic calcium channel blockers. The phenylalkylamines, the benzothiazepines and the 1,4-dihydropyridines, represented by verapamil, diltiazem and nifedipine, respectively, are the best known groups of these compounds. Although these three classes of drugs act at three discrete binding sites on the voltage-dependent slow calcium channels, it has been reported that some new classes of drugs modulate the channel activity by acting at other sites [215]. The L-type calcium channel is a large multimeric protein with five subunits [147]. It is likely that such a large complex will have many drug binding sites. The frequency- and voltage-dependent inhibitory effects of non-peptide opioids on voltage-dependent slow calcium channels are very similar to the effects produced by the organic calcium channel blockers on the voltage-dependent slow calcium channels. Thus, it would be interesting to see if these non-peptide opioids have direct or allosteric effects on the

organic calcium channel blockers' binding sites in skeletal muscle. Whether these opioids define a new site of action remains to be established (Fig. 27).

The effects of non-peptide opioids on the voltage-dependent slow calcium channels in frog skeletal muscle, presented in this study, were nonstereospecific and resistant to antagonism by naloxone. This indicated that a different type of 'opioid drug receptor' was involved. It would be important to determine whether the non-peptide opioids produce similar effects on mammalian skeletal muscles.

Loperamide, an opioid chemically related to meperidine, is clinically used as an anti-diarrhoeal agent. It has a calcium channel blocking effect in taenia coli muscle, which is only partially sensitive to antagonism by naloxone [217, 218]. This inhibitory effect of loperamide on calcium channels has been proposed to contribute to its anti-diarrhoeal effect [218]. It would be interesting to see whether loperamide produces a similar effect on calcium channels in skeletal muscle, and if so, to study the possible involvement of a nonstereospecific, naloxone-resistant receptor in such a response.

Non-opioid antitussive agents, such as dextromethorphan, noscapine, caramiphen have been reported to act through a receptor which is nonstereospecific and naloxone-resistant [1]. In addition, dextromethorphan has been reported to block voltage-dependent slow calcium channels in brain synaptosomes and cultured neural

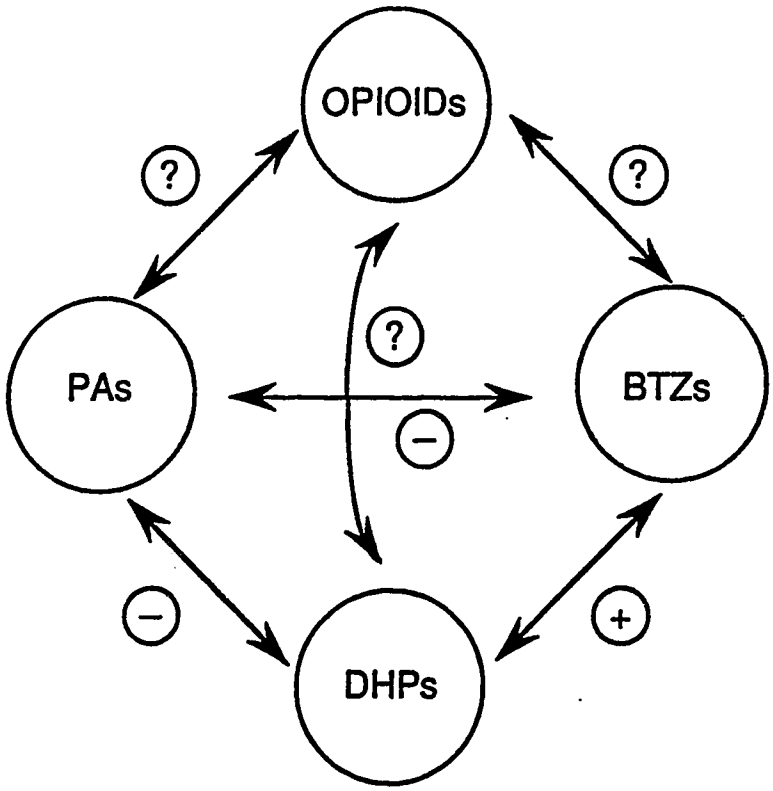

FIG. 27 Schematic arrangement of voltage-dependent slow calcium channel binding sites. The estabalished primary binding sites for 1,4 - dihydropyridines (DHPs), phenylalkylamines (PAs) and benzothiazepines (BTZs) are linked through a series of positive and negative heterotropic interactions (+ and -). Because the identity of the binding site of non-peptide opioids has not been established nor has the heterotropic relationship to other sites been established, this site has been depicted as independent.
(Note: Above figure was modified from ref. [215].)

(PC12) cells [213]. In some preliminary studies from our laboratory, we observed that dextromethorphan did block K^+ contractures in frog skeletal muscle. The inhibitory effects of dextromethorphan on K^+ contractures were observed in concentrations at which the twitch was potentiated. These effects are similar to the inhibitory effects of the non-peptide opioids on voltage-dependent slow calcium channels in skeletal muscle. Thus, it would be important to determine whether dextromethorphan and other antitussive agents produce such an effect through the nonstereospecific, naloxone-resistant receptor involved in the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels in frog skeletal muscle.

6. SUMMARY AND CONCLUSIONS

1. The effects of several opioid drugs were tested on the K^+ contractures of the frog toe muscles. These contractures are produced by the influx of extracellular calcium ions via the voltage-dependent slow calcium channels located in the t-tubules. Methadone and several other non-peptide opioids inhibited K^+ contractures in low concentrations. By contrast, various opioid peptides had little or no effect on these contractures.

2. The inhibition of K^+ contractures by non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone. This implied that the effects of non-peptide opioids are not mediated by any of the proposed opioid receptor subtypes, but are due to activation of nonstereospecific, naloxone-resistant opioid receptors.

3. The inhibitory effects of non-peptide opioids on the K^+ contractures were reversed by raising $[Ca]_o$ from 1.08 mM to 5 mM. This reversal may reflect competition for binding and/or an increase in electrochemical driving force for Ca^{++} influx through the unblocked slow calcium channels.

4. Non-peptide opioids, in concentrations used to block K^+ contractures had no effect on the K^+-induced membrane depolarization. These results indicated that the blocking effects of non-peptide opioids on K^+ contractures were not due to the inhibition of K^+-induced membrane depolarization.

5. These effective non-peptide opioids, in concentrations used to block K^+ contractures had no effect on caffeine contractures. These results indicate that non-peptide opioids neither deplete intracellular calcium stores nor inhibit the release of calcium from intracellular sarcoplasmic reticulum stores. This also indicates that the function of the internal contractile apparatus is not modified by the non-peptide opioids. Moreover, the non-peptide opioids tested were shown in a previous study to produce twitch potentiation or no effect on twitches, in the concentration range used to inhibit K^+ contractures.

6. These effective non-peptide opioids also inhibited Ca^{++}-dependent slow APs in frog skeletal muscle. These slow APs are produced by the influx of calcium ions through the voltage-dependent slow calcium channels. Inhibition of the Ca^{++}-dependent slow APs by the non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone. As was the case with the K^+ contractures, various opioid peptides produced little or no effect on these Ca^{++}-dependent slow APs.

7. The inhibitory effects of non-peptide opioids on the K^+ contractures were found to be frequency- and voltage-dependent. This inhibition could be accelerated (and also potentiated) by increasing the frequency of K^+ contracture tests, and by partial depolarization of the skeletal muscle

membrane in a chloride-free medium. These frequency- and voltage-dependent inhibitory effects are similar to the action of local anesthetics on the sodium channels, and can be interpreted within the framework of the modulated receptor hypothesis. These results indicate a preferential binding of the non-peptide opioids to their receptors in the open- and/or inactivated-state of the calcium channels. It is possible that the non-peptide opioids are shifting the steady-state inactivation curve towards more negative potentials. Such an effect would suggest that the non-peptide opioids preferentially block channels that are in the inactivated state. Another possible explanation is that the non-peptide opioids can reach their receptors inside the channels only when the channels are open. Calcium channels are continuously open in partially depolarized muscle and thus allow the effective opioids to reach their receptors in the channels more effectively than in the normally polarized cells.

8. In a vascular smooth muscle preparation, Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) has been reported to have calcium-agonist activity, that is resistant to antagonism by naloxone. However, DTALE had no effect on voltage-dependent slow calcium channels in frog skeletal muscle.

9. Haloperidol, which binds to σ-receptors with high affinity, inhibited voltage-dependent slow calcium channels in frog skeletal muscle. However, its effects were not specific to calcium channels. Also it inhibited twitches and

caffeine-induced contractures in the same concentration range. Haloperidol has also been reported to block sodium channels in the same concentration range.

10. It was concluded that several non-peptide opioids block the voltage-dependent slow calcium channels in frog skeletal muscle, thereby blocking K^+ contractures and Ca^{++}-dependent slow APs. This effect was nonstereospecific and naloxone-resistant. By contrast, various opioid peptides showed little or no effect on these channels. These results suggest that there are important differences between the effects of non-peptide opioids and natural endogenous ligands for opioid receptors.

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