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The effect of opiates on the secretion of transmitter from amphibian motor nerve terminals

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Abstract

The effects of dynorphin-A, dermorphine and morphine on the secretion of transmitter from the toad (*Bufo marinus*) motor nerve terminal have been determined. Intracellular recordings of miniature end plate potentials (m.e.p.p.s) and evoked end plate potentials (e.p.p.s) were used to estimate quantal content ($\overline{m}$) and binomial parameters p and n. Dynorphin-A, and to a lesser extent morphine, decreased ($\overline{m}$) while dermorphine had no significant effect on $\overline{m}$. Dynorphin-A (ED_{50} = 24 μM) was 21 times more potent then morphine (ED_{50} = 510 μM) in decreasing $\overline{m}$. The decrease in $\overline{m}$ produced by dynorphin-A and morphine was accompanied by a greater decrease in the variance (S^2) of number of quanta secreted per stimulation over the recording period. The decrease in $\overline{m}$ produced by dynorphin-A, and to a lesser extent by morphine, is probably mediated by the opiates acting on κ-opioid receptors.

Keywords: Neurotransmission; Opiate; Dynorphin-A; Morphine; Dermorphine; Motor nerve

1. Introduction

Opiates have been shown to reduce the amount of neurotransmitter secreted by nerve stimulation (for a review see Duggan and North, 1984). At the amphibian neuromuscular junction, high concentrations of morphine produced a decrease in the amount of acetylcholine secreted from cholinergic terminals during nerve stimulation (Frederickson and Pinsky, 1971). [Met^5]Enkephalin has also been shown to reduce the number of quanta secreted during sympathetic ganglion transmission (Hirai and Katayama, 1988) and modulate motor neurone activity in the spinal cord of the bullfrog *Rana catesbeiana* (Suzuki et al., 1987). At the amphibian neuromuscular junction, morphine decreases acetylcholine secretion either by binding to κ-opioid receptors (which are thought to be coupled to the potential-dependent calcium channels (McFadzean, 1988; Traynor, 1989) producing a reduction in the amount of Ca^{2+} influx into the terminal during nerve stimulation, or by binding with μ-opioid receptors (which have been shown to be coupled to potassium channels: Morita and North, 1982; North, 1986; North et al., 1987), hyperpolarizing the terminal and causing conduction failure of the action potential at branch points or along terminal branches. The inhibitory action of morphine on the amphibian nerve terminal is likely to occur via a decrease in the entry of extracellular Ca^{2+} during nerve stimulation, since [Met^5]enkephalin reduces the number of quanta secreted following local depolarisation of the terminal (Bixby and Spitzer, 1983). This study looks at the effects of κ- and μ-opioid receptor agonists on quantal content ($\overline{m}$) and its variance (S^2) in order to establish which of the opioid receptors is responsible for the decrease in acetylcholine secretion.

Motor nerve terminals innervating the toad iliofibularis muscle are composed of hundreds of release sites regularly spaced at about 1 μm intervals (Bennett et al., 1987). If, however, the extracellular calcium concentration ($[Ca^{2+}]_o$) is reduced, to minimise contraction, the variation in numbers of quanta secreted from impulse to impulse is not as large as would be expected if all release sites had equal opportunity to secrete a quantum of transmitter. Opiates such as dynorphin, which is found in relatively high concentrations in brain and spinal cord of *Bufo marinus* (Cone and

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Goldstein, 1982), or dermorphine found on the skin of frogs (Montecucchi et al., 1981), may act as modulators of quantal secretion by decreasing the probability of quantal secretion from release sites. This study investigates the effect of dynorphin-A, morphine and dermorphine on $\overline{m}$ and S^2, as well as the effect of an opioid receptor antagonist (naloxone) on quantal secretion.

2. Materials and methods

2.1. Housing of animals

Toads (*Bufo marinus*) ranging in length from 55 to 70 mm from tail to nose were collected from the Northern Table Lands of Queensland, Australia and used within 4 weeks of capture. During this period they were maintained in a room which was fitted with 15% ultraviolet lights, that were turned on for 16 h per day. The temperature of the room was maintained between 25 and 30°C and the animals were fed 2 times per week with a mixture of ground meat and fish brittle.

2.2. Tissue preparation

Animals were anaesthetised with tricaine methanesulphonate (MS222, 0.05%, Rural Chemical Industries Australia) and then killed by a cervical fracture. The right iliofibularis muscle with its nerve supply was dissected from its surrounding connective tissue and cut at its tendinous insertions. The muscle was then pinned to the bottom of the 3 ml capacity bath on Sylgard, with the point where the nerve enters the muscle facing up and stretched to approximately 110% of its resting length in the limb to form a flat parallelogram. The preparation was continuously perfused at the rate of 5 ml/min with a modified Ringer solution containing (mM): Na^+, 117.0; K^+, 3.0; Mg^{2+}, 1.2; Cl^-, 105.5; $H_2PO_4^-$, 1.3; HCO_3^-, 16.3; Ca^{2+}, 0.35–0.4; glucose, 7.8. The temperature of the bath was maintained between 13 and 15°C. The reservoir supplying the bath was continuously gassed with 95% O_2 and 5% CO_2, and the pH was maintained between 7.2 and 7.5.

2.3. Stimulation

The iliofibularis nerve was gently sucked into a pipette filled with the Ringer solution. A silver/silver chloride wire was then used to stimulate the nerve using square wave current pulses of 0.08 ms duration and 8 V amplitude. The nerve was stimulated continually at 0.2 Hz while searching for a focal recording of an end-plate potential (e.p.p.). When the microelectrode was optimally positioned stimulation was discontinued for 15 min before samples of e.p.p.s were taken. At least 200 nerve stimulations were recorded at 0.2 Hz for each intracellular impalement.

2.4. Recording

Intracellular recordings of the e.p.p. were obtained using microelectrodes filled with KCl (2 M) and having a resistance of between 30 and 50 MΩ. Focal recordings of the e.p.p. were obtained by placing the electrode about mid-length of the muscle fibre and noting the rate of rise of the e.p.p. If the rise time was less than 1.5 ms and the mean m.e.p.p. amplitude was greater than 0.7 mV the electrode was left in that position for the duration of the experiment. Two hundred e.p.p.s were recorded to construct each amplitude frequency histogram. More than 30 m.e.p.p.s were recorded to construct the amplitude frequency histogram which was used to determine quantal size. The average number of quanta secreted per impulse (quantal content, $\overline{m}$) during the 200 impulses was determined by dividing the mean amplitude of the e.p.p.s by the mean amplitude of the m.e.p.p.s.

2.5. Storage and analysis of data

An IBM-AT computer and p-Clamp software (Axon Instruments) were used to store traces each lasting 10 ms following triggering by the stimulator. The signals were filtered at 5 kHz. For each recording, 200 traces were collected, stored on hard disc and then transferred to floppy disc for long-term storage. The signals were analysed using p-Clamp software (modified by Dr. F. Edwards). Measurements of the amplitude, latency from the start of the trace, rise time, and rate of rise of the e.p.p.s and m.e.p.p.s were taken and stored. Histograms of any of these measurements versus frequency could then be constructed from these.

Estimates of $\overline{m}$, the mean probability of quantal secretion (p) and the maximum number of release sites participating in secretion during the 200 nerve stimulations (n) were made using the equations described in Robinson (1976).

2.6. Drugs

Stock solutions of drugs were made and aliquoted into portions. Dynorphin-A (Sigma) was dissolved in 40 μM acetic acid (pH about 5) at a concentration of 1 μM and aliquoted into 0.1 ml portions. Dermorphine (Sigma) was prepared in the same way. All vials were then stored at −70°C until needed for the experiment. Morphine hydrochloride and naloxone hydrochloride were a gift from the Department of Pharmacology, University of Sydney. They were dissolved in distilled water and stored below 4°C. The tissue was then bathed in Ringer containing the drug for about 40 min before recordings of the e.p.p.s or m.e.p.p.s were taken.

3. Results

3.1. The effect of opiates on quantal secretion

The effects of morphine, dermorphine and dynorphin-A on quantal secretion were compared. Morphine is known to be a potent μ-opioid receptor agonist but a poor κ-opioid receptor agonist. At the toad motor-nerve terminal, morphine (320 μM) reduced $\overline{m}$ by about $21 \pm 5\%$ (mean $\pm$ S.E.M., $n = 6$). Dermorphine is a fairly specific μ-opioid receptor agonist (Amiche et al., 1989; Charpentier et al., 1991): at a concentration of 83 μM and 166 μM it decreased $\overline{m}$ by $8 \pm 4\%$ ($n = 5$) and $9 \pm 7\%$ ($n = 3$) respectively; this affect was not significant. On the other hand, the specific κ-opioid receptor agonist, dynorphin-A (Gairin et al., 1984, 1986; McFadzean, 1988; Traynor, 1989), at a concentration of 13 μM reduced $\overline{m}$ by about $30 \pm 5\%$ ($n = 12$, Fig. 1). This effect was readily reversed by washing the preparation with drug-free Ringer solution (Fig. 1). The log dose vs. percentage inhibition in $\overline{m}$ relationship for both dynorphin-A- and morphine-treated preparations was determined to compare their relative potencies (Fig. 2). The effective dose producing 50% inhibition in $\overline{m}$ (ED_{50}) for dynorphin-A and morphine was 24 μM and 510 μM respectively. Thus dynorphin-A was 21 times more potent than morphine in decreasing the number of quanta secreted during nerve stimulation.

In the presence of naloxone (10 μM), morphine (320 μM) was still able to reduce $\overline{m}$ by $17 \pm 9\%$, $n = 6$, but this was not statistically significant compared to its effect in the absence of naloxone ($P < 0.3$). Naloxone (10 μM) also had no significant effect ($P < 0.3$) on the dynorphin-A-induced decrease in $\overline{m}$ ($23 \pm 6\%$, $n = 9$ compared to $30 \pm 5\%$). Naloxone (a good μ-opioid receptor antagonist) at a concentration of 10 μM was shown to have very little antagonism to the effects of dynorphin-A and morphine. Naloxone at 50 μM reduced the dynorphin-A-induced decrease in $\overline{m}$ from $30 \pm 5\%$ to $11 \pm 9\%$ ($n = 3$) and the morphine-induced decrease in $\overline{m}$ from $21 \pm 5\%$ to $12 \pm 8\%$ ($n = 6$). This is consistent with the idea that it is κ-opioid-like receptors and not μ-opioid receptors that mediate opiate effects on $\overline{m}$. Exposure of the motor nerve terminals to naloxone (10 μM) alone resulted in a small increase in $\overline{m}$ ($5 \pm 12\%$ increase, $n = 8$). At higher concentrations (50 μM) naloxone increased $\overline{m}$ by $28 \pm 30\%$ ($n = 7$).

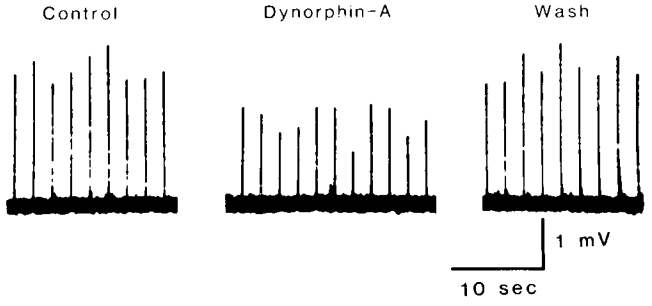

Fig. 1. Sample recordings of the end plate potential from a nerve terminal before and during dynorphin-A (13 μM) treatment and after washing the drug out. Between 9 and 11 consecutive evoked end plate potentials are shown. The stimulation frequency was 0.25 Hz and the extracellular Ca^{2+} concentration was 0.4 μM.

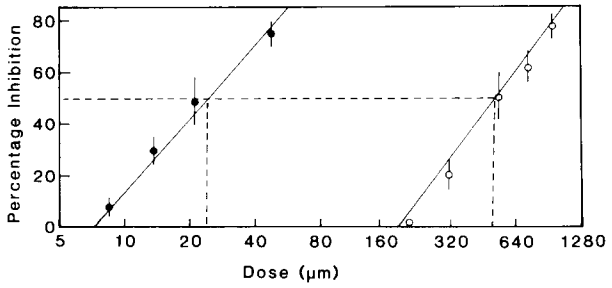

Fig. 2. Percentage inhibition of $\overline{m}$ vs. log dose of dynorphin-A and morphine. The inhibitory effects of dynorphin-A (filled circles) and morphine (open circles) are compared. Dots represent the means of at least 5 terminals and bars represent S.E.M. Lines of best fit were determined by linear regression. For dynorphin-A the slope of the line was 1.14 and the correlation coefficient was 0.82, while for morphine slope was 1.33 and correlation coefficient was 0.90. Dashed lines indicate the doses which produced 50% inhibition in $\overline{m}$, for dynorphin-A it is equal to 24 μM and for morphine it is equal to 510 μM.

3.2. The effect of opiates on quantal size

At higher concentrations of naloxone (≥ 100 μM), an estimate of the $\overline{m}$ could not be made since naloxone in a dose-dependent manner decreased the mean amplitude of the m.e.p.p.s. Naloxone (10 μM) decreased the mean m.e.p.p. amplitude by about $19 \pm 8\%$ ($P < 0.05$, $n = 5$), while at 50 μM the mean m.e.p.p. amplitude decreased by about $41 \pm 6\%$ ($P < 0.01$, $n = 5$). Dynorphin-A (13 μM), morphine (320 μM) and dermorphine (83 μM) had no significant effect on m.e.p.p. amplitude or frequency. Dynorphin-A decreased the size of m.e.p.p.s by $11 \pm 6\%$ ($n = 9$), morphine decreased the size of m.e.p.p.s by $9 \pm 14\%$ ($n = 6$) and dermorphine increased m.e.p.p. amplitude by $10 \pm 21\%$ ($n = 4$). All these changes were not significant ($P < 0.1$); also the frequency of m.e.p.p.s did not significantly change (compare dashed line histograms in Fig. 3). The frequency (Hz) of spontaneous quantal secretions was in one study 0.07 for control, 0.09 in the presence of morphine, 0.1 after washing, and in another study 0.08 for control, 0.08 in the presence of dynorphin-A, 0.11 after washing (Fig. 3).

3.3. The effect of dynorphin-A and morphine on the probability of quantal secretion

Comparing the amplitude-frequency distributions for the m.e.p.p.s of control, morphine-treated and post-

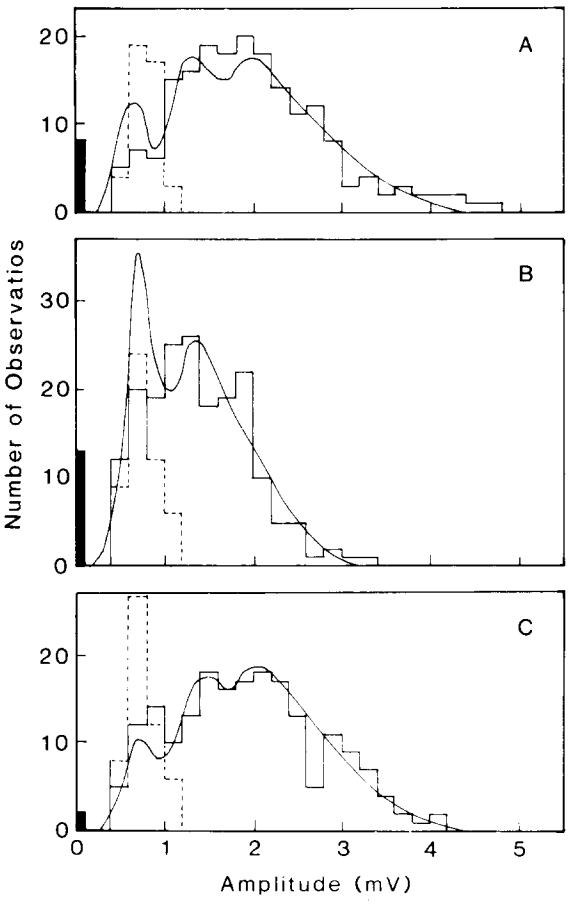

Fig. 3. Amplitude of e.p.p.s vs. number of observations histograms for a dynorphin-A-treated terminal. A: control; B: dynorphin-A (24 μM) treated; C: after washing the drug from the preparation. Dashed lines indicate the amplitude vs. number of observation histograms of the m.e.p.p.s, while solid lines indicate the amplitude vs. number of observation histograms of the e.p.p.s. Black bars at zero amplitude indicate the number of failures in 200 nerve stimulations. Curve fit is the prediction fits according to binomial statistics. The binomial parameters of $\overline{m}$, n and p are given in Table 1. The P values for a χ^2 test for 'goodness of fit' of the binomial distributions to the histograms in A, B and C as well as all the binomial distributions described in Table 1 were between 0.05 and 0.1. As expected given the large p values, Poisson predictions (not shown) did not fit the data.

washout preparations demonstrated that there was no change in either their mean amplitude or their variance (Table 1). Morphine did, however, decrease $\overline{m}$ and S^2 (Table 1). The decrease in S^2 was greater than the decrease in $\overline{m}$, indicating that p increased while binomial parameter n decreased (Table 1).

Similarly dynorphin-A had no effect on the mean amplitude or variance of the m.e.p.p.s (Fig. 3 and Table 1) but caused a decrease in $\overline{m}$ with a slightly greater decrease in S^2. Again since the decrease in S^2 was slightly greater than the decrease in $\overline{m}$, p calculated from binomial statistics increased while parameter n decreased giving an overall decrease in $\overline{m}$ (Fig. 3 and Table 1). Dermorphine produced a 8% decrease in $\overline{m}$ and a 4% decrease in S^2. Neither of the dermorphine changes was statistically significant.

A summary of the effect of dynorphin-A on the binomial parameter $\overline{m}$ and p can be seen in Fig. 4A and B. Dynorphin-A produced a decrease in $\overline{m}$ for 17 of the 18 terminals studied (Fig. 4A). An increase in the binomial parameter p was seen in 15 of the 18 terminals studied (Fig. 4B). In the 21 terminals studied morphine (320 μM) decreased $\overline{m}$ by $32 \pm 6\%$ (mean $\pm$ S.E.M.) and increased p by $21 \pm 5\%$.

4. Discussion

4.1. The effect of opiates on quantal size

The effects of dynorphin-A, dermorphine, morphine and naloxone on postsynaptic receptors was investigated by comparing the amplitude of m.e.p.p.s before and after drug treatment. Opiates have been shown to inhibit the action of 16S acetylcholinesterase (Haynes and Smith, 1982). A slight increase in the size of quanta was expected; however, with all the opiate agonists tested none showed any significant change in the size of quanta. In most preparations, morphine ($\geq$ 320 μM) produced a slight increase in the duration of the m.e.p.p.s and e.p.p.s. In addition there was an increase in the noise shortly after nerve stimulation. These changes are probably induced by the anti-acetylcholinesterase action of morphine. Since the opiate antagonist, naloxone, decreased m.e.p.p. size in a dose-dependent manner, sufficiently high doses of naloxone could not be used to overcome the effects of dynorphin-A or morphine while still being able to

Table 1
The effect of morphine (500 μM) and dynorphin-A (24 μM) on the amplitude and variance [a] of m.e.p.p.s and e.p.p.s, $\overline{m}$, p and n

Treatment	m.e.p.p.	e.p.p.	$\overline{m} \pm$ S.E.	$p \pm$ S.E.	$n \pm$ S.E.
Control	0.62 ± 0.04	2.98 ± 1.02	4.38 ± 0.31	0.55 ± 0.07	8.78 ± 1.35
Morphine	0.60 ± 0.04	0.97 ± 0.27	1.60 ± 0.12	0.65 ± 0.05	2.46 ± 0.31
Wash	0.59 ± 0.04	2.92 ± 0.82	4.96 ± 0.33	0.63 ± 0.06	7.87 ± 1.04
Control	0.68 ± 0.03	1.85 ± 0.78	2.71 ± 0.16	0.44 ± 0.07	6.22 ± 1.03
Dynorphin-A	0.67 ± 0.04	0.94 ± 0.30	1.41 ± 0.09	0.61 ± 0.05	2.33 ± 0.24
Wash	0.67 ± 0.03	1.88 ± 0.72	2.81 ± 10.16	0.51 ± 0.06	5.55 ± 0.83

At least 200 recordings were taken for each treatment. [a] Mean amplitude ($\overline{m}$V) ± variance ($\overline{m}$V^2).

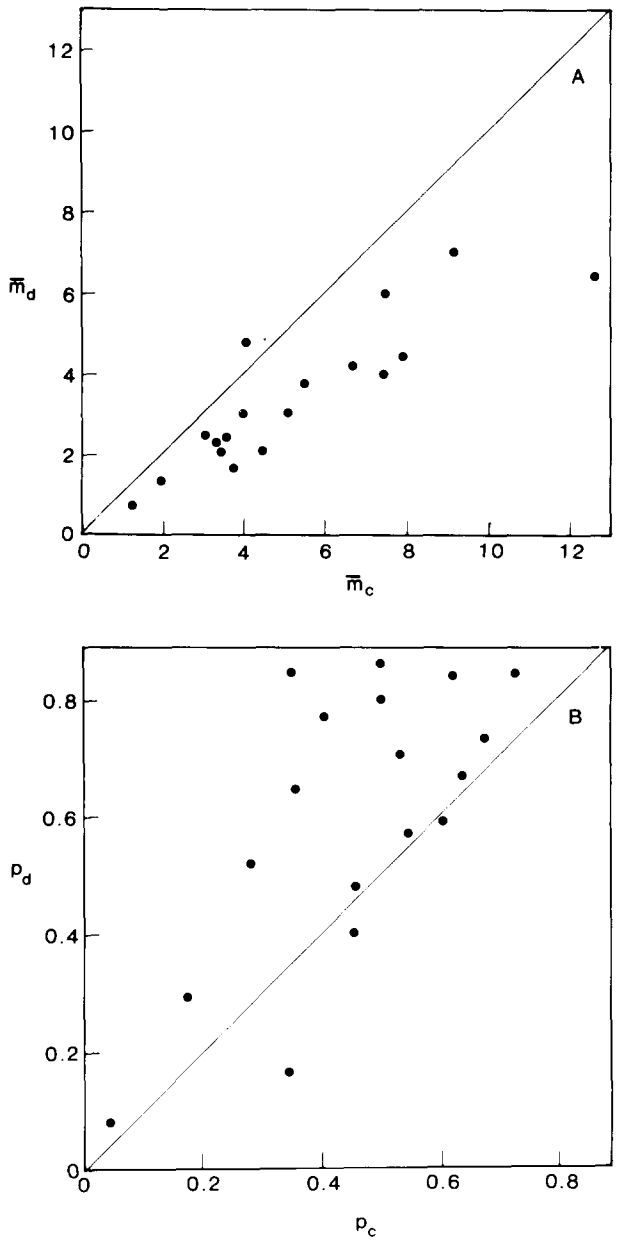

Fig. 4. The effect of dynorphin-A on the binomial parameters $\bar{m}$ and p. Each filled circle represents a terminal chosen for study from each muscle preparation. Points falling on the diagonal lines represent no change, points falling below this line represent a decrease, while points found above this line indicate an increase. In A, the quantal content before dynorphin-A treatment ($\bar{m}_c$) is plotted against the quantal content following 30–40 min of dynorphin-A (13 μM) treatment ($\bar{m}_d$). Note that most of the points fall below the diagonal line. In B, the probability of quantal secretion before dynorphin-A treatment (p_c) is plotted against the probability of quantal secretion following 30–40 min of dynorphin-A treatment (p_d). Note that most of the points fall above the diagonal line.

record m.e.p.p.s. For the same reasons the possibility that endogenous opiates suppress quantal secretion during normal neurotransmission could not be properly tested with naloxone. Nevertheless a slight increase in $\bar{m}$ was observed after administration of naloxone.

4.2. The effect of opiates on the evoked secretion of quanta

Frederickson and Pinsky (1971) demonstrated that morphine in high concentrations decreases the amount of acetylcholine secretion from amphibian motor nerve terminals following nerve stimulation. This decrease occurs even after local depolarisation of the terminal (Bixby and Spitzer, 1983). Hence it is unlikely that the decrease in quantal content occurs as a consequence of failure of the action potential to propagate along terminal branches. A more likely mechanism might involve changes in the entry of extracellular Ca^{2+} into the nerve terminal following nerve stimulation or the process involving vesicle secretion. Since the rate of spontaneous quantal secretion was not significantly altered even at concentrations of opiates that reduced quantal content to less than half control values, it is unlikely that opiates affect the vesicle secretion process.

Given that dynorphin-A was 21 times more potent then morphine in decreasing quantal content and since dermorphine showed very little effect on quantal secretion, it is likely that opiates are decreasing evoked quantal secretion by acting on presynaptic κ-opioid receptors. There is some suggestion that they are δ-opioid receptors (Bixby and Spitzer, 1983) which affect the voltage-dependent calcium channels. The opiate-induced decrease in transmitter secretion is not mediated by failure of action potential propagation (Bixby and Spitzer, 1983); indirect evidence suggests that a more likely mechanism involves the inhibition of voltage-dependent calcium channels (Jessell and Iversen, 1977; MacDonald and Nelson, 1978; Bornstein and Fields, 1979; Bennett and Lavidis, 1980; Bixby and Spitzer, 1983; Michaelson et al., 1984). In this study dynorphin-A and morphine decreased evoked quantal secretion without increasing the frequency of failures to secrete quanta. The opposite result would be expected if conduction failure was occurring.

It is possible that the decrease in quantal content and slightly greater decrease in the variance in number of quanta secreted during the stimulation period, is produced by a decrease in the number of release sites participating in quantal secretion when dynorphin-A or morphine is administered. The decrease in number of release sites participating in quantal secretion would be expected to occur if (1) some terminal branches were more susceptible to opiates, or (2) there is differential opiate sensitivity of release sites, or (3) opiates uniformly decrease the probability of quantal secretion resulting in the relative silencing of low-probability release sites. To distinguish among these possibilities requires that recordings from small groups of release sites along nerve terminal branches be compared before and after opiate treatment.

4.3. Are endogenous opiates affecting quantal secretion?

The variance in the number of release sites showing activity during this period is very small if one considers the total number of release sites. It is possible that some co-transmitter and/or hormonal neuroregulators decrease the probability of quantal secretion from the majority of release sites. Such modulation has been reported in two studies. At the release sites of the amphibian motor nerve terminal the probability of quantal secretion is modulated during development (Bennett and Lavidis, 1988) and by seasonal factors (Bennett and Lavidis, 1992). The identity of endogenous factors producing this modulation is unknown. The effect of adenosine which is co-released with acetylcholine in the form of ATP (Silinsky, 1975) has been determined (Bennett et al., 1991).

In the present study evidence for the likely existence of κ-opioid-like receptors on toad (*Bufo marinus*) motor nerve terminals was provided. When activated by the exogenous administration of dynorphin-A these receptors produce a decrease in quantal content with a greater decrease in the variance of number of quanta secreted per impulse. To determine the possible physiological role of these opioid receptors the source of endogenous opiates which act on these will need to be identified.

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