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Depression of Frog Isolated Spinal Cord by Morphine and Antagonism by Tetracosactin (38150)

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Introduction. Tetracosactin ($ACTH_{1-24}$), the first amino acid residue of adrenocorticotropic hormone (ACTH), has been shown to possess a pharmacologic spectrum of activity similar to ACTH (1). More recently it has been observed that $ACTH_{1-24}$ antagonizes the depressant action of morphine (MS) on the monosynaptic reflex of the cat spinal cord (2). Indirect evidence suggested the hypothesis that this antagonism was not a result of the corticotropic action of $ACTH_{1-24}$. The present study was undertaken to test that hypothesis, and to test the additional hypothesis that $ACTH_{1-24}$ can act directly on neural tissue to antagonize MS. To accomplish this, use was made of the fact that frog spinal cord can be maintained *in vitro,* isolated from other body systems, for the study of monosynaptic activity (3). The initial step in this study was to examine the influence of MS on this preparation, and then to determine the specificity of MS-induced changes by studying the interaction between MS and its specific antagonist naloxone (N). Further, since calcium has been reported to antagonize MS (4), it was of interest to determine the influence of $CaCl_2$. During the course of these preliminary experiments, it seemed that the magnitude of the MS-induced changes was related to the season of the year, leading to an investigation of this as a possible variable. Although frog spinal cord remains viable *in vitro* for reasonable periods of time, attempts were made to prolong its utility by reducing temperature. Having characterized the preparation, the primary objective was investigated, i.e., the actions of $ACTH_{1-24}$ were studied alone, and in the presence of MS.

Materials and Methods. Frogs (Rana pipiens) weighing 30–50 g were maintained at room temperature (approximately 22°) until ready for use. Spinal cords were isolated and used as described by Brookhart, Machne and Fadiga (3) with the following modifications:

1. The composition of the Ringer's solution was: NaCl 73.25 m*M*, $NaHCO_3$ 35.0 m*M*, KCl 3.13 m*M*, $Na_2HPO_4 \cdot H_2O$ 0.59 m*M*, $CaCl_2 \cdot 2H_2O$ 0.5 m*M*, and glucose 16.18 m*M* per liter, and equilibrated with 95% O_2–5% CO_2. During the course of each experiment the solution was kept under constant pressure of the gas mixture (50 mmHg) and superfused over the cord (1 ml/min) at 18.0 ± 0.5°. Under these conditions the pH of this solution remained constant (7.4).

2. A lumbar dorsal root was cut at its junction with its corresponding ventral root, and each root was placed in a series of pools of Ringer's solution separated by petroleum jelly. Platinum wire electrodes were placed in appropriate pools to provide for stimulation of the dorsal root or recording from the ventral root. The recording electrodes were positioned in distal pools containing the ventral root such that the evoked response did not include the d-c potential generated by the spinal cord. A third pair of platinum wire electrodes was positioned 1 mm apart in contact with the lateral aspect of the cord several segments rostral to the attachments of the spinal roots.

Stimulation of the lateral column 80 msec after stimulating the dorsal root gives rise to a monosynaptic ventral root response (MVR) (3), the potential that was studied in these

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experiments. Stimuli were biphasic, 0.5 msec in duration and of a voltage sufficient to evoke maximal amplitude of MVR. Once determined the stimulus parameters remained unchanged and the paired stimuli were delivered through stimulus isolation units every 5 sec throughout each experiment. The monosynaptic potentials evoked in the ventral root by lateral column stimulation (MVR) were amplified using conventional electrophysiological techniques, displayed on an oscilloscope, and photographed. Later the photographs were projected onto graph paper and the amplitudes measured.

The sequence of recording and administration of test substances was the same in each experiment. After approximately 1 hr of stimulation, MVR had stabilized and the experiment commenced (time 0 min). At 30 min, the superfusing fluid was switched to Ringer's solution containing the test substance, combined test substances or the vehicle. Thirty min later (time 60 min), the fluid superfusing the cord was changed back to ordinary Ringer's solution. After a washout period lasting 30 min, the experiment was terminated (time 90 min). A series of ten traces was recorded at each of the following times: 0, 15, 30, 60 and 90 min. The mean of the amplitudes of the MVR's recorded during the control period was calculated separately for each frog. The mean of each subsequent series of 10 traces was then expressed as a percent of the control mean for each separate experiment. The differences between the various treatment groups were compared either at 60 min (end of 30 min treatment), or at 90 min (end of 30 min washout) using the Student's *t* test.

Three studies were performed, and the treatments randomized within each study.

The first study was to determine the effects of MS and of its antagonist, naloxone (*N*) on MVR. In addition, the influence of seasonal variation on the response of MVR to MS was estimated by comparing the results of this study, in which spring frogs were used, with results obtained using late-fall frogs.

The second study was to determine the influence of MS on MVR, but in the presence of *N*, of increased $CaCl_2$ (2.0 m*M*), or of low temperature superfusate (13.0 ± 0.5°) (low temp). Appropriate controls in this study included an evaluation of the influence of *N*, of increased $CaCl_2$, or of low temp individually on MVR. When the influence of low temp was tested the temperature of the superfusion fluid was maintained at 13.0 ± 0.5° during the total experiment.

The third study was also to ascertain the influence of MS on MVR, but in the presence of $ACTH_{1\text{-}24}$ (2 or 20 μg/ml). Appropriate controls in this study included an evaluation of the influence of the effects of each concentration of $ACTH_{1\text{-}24}$ when it was given alone.

Each of the three studies was further controlled by determining the alterations of MVR across time when plain Ringer's solution was given under identical conditions. These controls were also randomly distributed within each study.

Results. The results of the first study are presented in Table I. After 30 min exposure

TABLE I. Effects of Various Concentrations of Morphine (MS) or Naloxone (N) on Monosynaptic Potentials Evoked in the Ventral Root by Stimulation of the Later Column of the Frog Isolated Spinal Cord.

Treatment Group	Number of cords	% of Control Amplitude Treatment (30 min)	 Washout (30 min)
A Ringer	6	105.8 ± 3.5[a]	98.8 ± 6.4
B Ringer + MS ($10^{-10}M$)	3	101.3 ± 10.4	90.7 ± 11.2
C Ringer + MS ($10^{-7}M$)	3	107.0 ± 6.7	104.3 ± 7.3
D Ringer + MS ($10^{-4}M$)	3	57.0 ± 13.2[b]	85.0 ± 19.1
E Ringer + N ($10^{-10}M$)	3	97.0 ± 10.4	92.0 ± 13.7
F Ringer + N (10^{-7} M)	3	83.3 ± 13.7	80.3 ± 15.8
G Ringer + N ($10^{-4}M$)	3	103.7 ± 6.2	104.7 ± 4.4

[a] Mean ± S.E.

[b] $P<0.01$ for D vs A, $P<0.05$ for D vs B, C, E, or G.

to MS $10^{-4}M$, the amplitude of MVR was reduced compared to that of control group A ($P < 0.01$). The amplitude of MVR following exposure to MS $10^{-4}M$ was also lower than those of cords superfused either with MS $10^{-10}M$ ($P < 0.05$) or $10^{-7}M$ ($P < 0.05$). Neither of the lower two concentrations of MS nor any of the concentrations of N caused significant alteration of MVR when compared to control group A ($P > 0.05$).

After 30 min washout, none of the treatment groups differed significantly from each other ($P > 0.05$ in each case).

In experiments using Ringer's solution alone, MVR at 60 min was similar for spring (105.8 ± 3.5) ($n = 6$) and fall (105.6 ± 5.1) ($n = 5$) frogs. In both spring and fall frogs, MS $10^{-4}M$ produced a significant depression of MVR after 30 min exposure ($P < 0.05$ in each case). The reduction in spring frogs ($n = 3$) was to 57 ± 13.2; whereas, that of fall frogs ($n = 5$) was to 92.5 ± 4.1. This difference between spring and fall frogs was significant ($P < 0.05$). After 30 min washout, the MVR of fall frogs was not significantly different from that of spring frogs.

The results of the second study are shown in Table II. After 30 min, MS $10^{-4}M$ caused greater suppression of MVR than did any of the other treatments ($P < 0.05$ for each comparison). The MVR of the Ringer treatment was lower than that of the cords exposed to MS plus N, low temp, or elevated $CaCl_2$ ($P < 0.05$ for each).

Thirty min after washout, the MVR of the MS-treated cords appeared to be returning to predrug levels, but was still reduced relative to the MVR of cords exposed to Ringer alone, elevated $CaCl_2$ plus MS, elevated $CaCl_2$ alone, MS plus low temp, or low temp alone ($P < 0.05$ for each). The MVR of Ringer-treated control cords remained lower than that of cords exposed to elevated $CaCl_2$ ($P < 0.05$) or low temp ($P < 0.01$). In addition, the MVR of cords treated with MS plus low temp was lower than that of cords exposed to elevated $CaCl_2$ ($P < 0.05$).

To examine further the influence of temperature and of $CaCl_2$ on MVR, data from appropriate groups in the second study were combined and subjected to factorial analysis. Comparison of groups A+B versus E+F (Table II) revealed that MVR was lower at 18° than at 13° after 30 min treatment ($P < 0.01$). Significant interaction of temperature with MS was observed after 30 min exposure to the drug ($P < 0.05$). Comparison of groups A+B versus G+H (Table II) revealed that MVR was lower ($P < 0.01$) after 30 min exposure to 0.5 mM $CaCl_2$ than to 2.0 mM $CaCl_2$. After 30 min washout, this difference was no longer evident ($P > 0.05$). Interaction of $CaCl_2$ with MS was significant ($P < 0.05$) after 30 min exposure to the drug. Thus, as indicated by these analyses, the capacity of increased $CaCl_2$ or of lowered temperature to facilitate synaptic transmission may explain part, but not all, of their ability to antagonize MS.

The results of the third study are presented

TABLE II. Effects of Naloxone (N), Decreased Temperature, or Increased Calcium Concentration on Morphine (MS)-Induced Suppression of Monosynaptic Potentials Evoked in the Ventral Root by Stimulation of the Lateral Column of the Frog Isolated Spinal Cord.

Treatment group	Number of cords	% of Control Amplitude	
		Treatment (30 min)	Washout (30 min)
A Ringer	4	90.4 ± 0.9[a]	90.2 ± 1.0
B Ringer + MS ($10^{-4}M$)	4	70.8 ± 3.8[b]	76.1 ± 3.9
C Ringer + MS ($10^{-4}M$) + N($10^{-4}M$)	4	103.4 ± 5.9	87.9 ± 16.3
D Ringer + N ($10^{-4}M$)	4	89.4 ± 4.5	101.4 ± 15.3
E Ringer + Low Temp. (13 °)	4	99.5 ± 3.0	96.1 ± 1.1
F Ringer + MS ($10^{-4}M$) + Low Temp. (13 °)	4	93.3 ± 3.4	88.2 ± 1.7
G Ringer + $CaCl_2$ (2.0 mM)	4	97.8 ± 3.3	96.7 ± 2.7
H Ringer + MS ($10^{-4}M$) + $CaCl_2$ (2.0 mM)	4	92.4 ± 3.8	96.2 ± 4.9

[a] Mean ± S.E.

[b] $P<0.05$ for B vs A, C, D, E, F, G, or H.

TABLE III. Effects of $ACTH_{1-24}$ and $ACTH_{1-24}$ plus Morphine (MS) on Monosynaptic Potentials Evoked in the Ventral Root by Stimulation of the Lateral Column of the Frog Isolated Spinal Cord.

Treatment Group	Number of cords	% of Control Amplitude	
		Treatment (30 min)	Washout (30 min)
A Ringer	5	100.9 ± 8.1[a]	99.6 ± 10.4
B Ringer + MS ($10^{-4}M$)	5	68.1 ± 5.7[b]	81.6 ± 14.1
C Ringer + MS ($10^{-4}M$) + $ACTH_{1-24}$ (2 μg/ml)	5	91.7 ± 6.2	91.2 ± 5.9
D Ringer + $ACTH_{1-24}$ (2 μg/ml)	5	101.4 ± 8.8	92.6 ± 9.6
E Ringer + MS ($10^{-4}M$) + $ACTH_{1-24}$ (20 μg/ml)	5	114.4 ± 18.7	94.3 ± 8.4
F Ringer + $ACTH_{1-24}$ (20 μg/ml)	5	103.8 ± 8.7	93.0 ± 8.4

[a] Mean ± S.E.

[b] $P < 0.01$ for B vs A, C, or E; $P < 0.05$ for B vs D, or F.

in Table III. As in the preceding studies, after 30 min exposure to MS $10^{-4}M$, MVR was reduced compared to the Ringer control group ($P < 0.01$). After 30 min exposure to MS $10^{-4}M$ in the presence of either 2 or 20 μg/ml $ACTH_{1-24}$, MVR was not reduced compared to that of the Ringer control group ($P > 0.05$). Exposure to either of these concentrations of $ACTH_{1-24}$ alone did not significantly alter the MVR as compared to the control group.

Thirty min after washout, the MVR's of the various treatment groups were not significantly different from each other ($P > 0.05$ for each comparison).

Discussion. The data presented demonstrate that MS (10^{-4}) depressed MVR of frog spinal cord when the cord is separated from other parts of the nervous system and of the body. This action of MS appears to be specific and to occur at the level of the synapse. It is likely specific since it is antagonized by *N* in concentrations which *per se* do not alter MVR. It likely occurs at the level of the synapse because, although MS ($10^{-4}M$) depresses nerve conduction of frog nerves which are tetanically stimulated (5), it does not do so when the nerves are stimulated at a frequency of 1 or 5 hz (Krivoy, unpublished observation). It is of interest that the concentration of MS which reduced MVR is similar to that reported by Angelucci (6) to depress frog spinal cord *in situ,* suggesting that the frog is relatively insensitive to MS when compared to higher species. The reason for this difference in sensitivity is not clear. It may reflect several factors. One of these is the fact that the synapse involved may be insensitive to MS. A second reason is that if the frog spinal cord responds in a manner similar to that of the cat (7) the supramaximal, low frequency stimulation used in the present studies would not favor observation of MS-induced depression.

The observation that spinal cords from spring frogs show greater suppression on exposure to MS than do fall frogs is in keeping with the observation of Smith (8), and should be borne in mind when data from this species are compared or pooled. The observation that increased $CaCl_2$ antagonizes MS is in keeping with the findings of Kakunaga, Kaneto and Hano (4). Although this may be a consideration in elucidating the mechanism by which MS acts, its importance to this communication resides in the fact that $CaCl_2$, an important constituent of Ringer's solution, can also be an impediment to using the frog as a model. The reason for studying the influence of temperature on the actions MS was to extend the viability of the spinal cord well beyond the limits necessary to perform the present study. The fact that decreasing temperature prevented the actions of MS is difficult to explain, but suggests a metabolic step is involved in this action of MS.

The first two studies showed that MS has a specific, direct action on frog spinal cord, and illustrates the importance of certain conditions in studying this action. The results obtained in the third study show that $ACTH_{1-24}$ can antagonize the depressant action of MS on MVR. This observation is consistent with the findings of Krivoy *et al.* (2) that $ACTH_{1-24}$ antagonizes the inhibitory action of MS on the segmental reflex of the decerebrate spinal cat. More importantly, the present results demonstrate that the antagonism can occur independent of other body systems, and consequently, that $ACTH_{1-24}$ has a direct action on the nervous system. The latter inference supports the conclusions of others. DeWied (9), examining the relationship between chemical structure and biological activity, concluded that the capacity of $ACTH_{1-24}$ to facilitate acquisition of the conditioned avoidance response was also due to its direct actions on neural tissue.

Summary. The purpose of these experiments was to determine if tetracosactin ($ACTH_{1-24}$) will antagonize the actions of morphine (MS) on neural tissue isolated from other body systems. The monosynaptic ventral root response (MVR) of frog isolated spinal cord *in vitro* was used to test this question. In this preparation MS produces a depression of MVR which is antagonized by naloxone, by $CaCl_2$, and by decreased temperature. $ACTH_{1-24}$ did not alter MVR, but did antagonize the actions of MS.

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