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Atropine Sulfate Increases Pituitary Responses to Stress[1,2]

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KANT, G J , L LANDMAN-ROBERTS, T EGGLESTON AND J L MEYERHOFF *Atropine sulfate increases pituitary responses to stress* PHARMACOL BIOCHEM BEHAV 26(3) 619–623, 1987 —The effects of atropine sulfate pretreatment on pituitary indices of stress response were examined Pituitary cyclic AMP and plasma prolactin increases following 15 min of acute stress were used as measures of stress response Over a range of doses (0, 5, 10, 30 and 60 mg/kg), pretreatment with atropine sulfate increased the measured stress responses to footshock but had little or no effect on resting or non-stressed levels of the substances measured. The effects of atropine on response to immobilization were tested only at 5 mg/kg At this dose, atropine sulfate, but not methylatropine nitrate, increased pituitary cyclic AMP response to immobilization stress demonstrating that the potentiation of the pituitary cyclic AMP stress response was not limited to footshock stress and suggesting that this effect of atropine was central rather than peripheral Neither atropine nor methylatropine pretreatment at this dose potentiated prolactin response to immobilization stress

Atropine Pituitary Stress Cyclic AMP Prolactin

OUR laboratory is engaged in a long-term effort to elucidate biochemical mechanisms that underlie observable physiological and behavioral responses to stressors. As part of these studies, we have studied the effects of acute exposure to stressors on levels of pituitary cyclic AMP and on the release of pituitary and adrenal hormones [8–13] Acute stress increases levels of pituitary cyclic AMP *in vivo* and this increase is related to the subsequent release of pituitary hormones In order to determine which neurotransmitters or releasing factors might be involved in the regulation of these stress responses, we conducted several experiments in which antagonists of putative regulators were injected prior to stress initiation. In a preliminary experiment, pretreatment of rats with atropine sulfate, 15 min prior to intermittent footshock, markedly increased hormonal and pituitary cyclic AMP responses to this stressor [16]

This preliminary finding was of particular interest to our laboratory because atropine sulfate is currently fielded in the U.S. Army as an antidote to nerve agent poisoning (nerve agents are cholinesterase inhibitors). Antidotes also might be mistakenly injected when no nerve agent exposure has actually occurred, especially under conditions of extreme stress. In the civilian community, atropine is similarly used clinically to treat victims of organophosphate pesticide exposure [28] Interactions between atropine and other factors likely to be present (e.g., stress) during conditions in which an antidote might be required may be important in determining appropriate dosages or predicting side effects of atropine injection For these reasons, we performed the series of experiments described in this report to further characterize the potential interaction between atropine and stress.

In order to assess stress response, we measured two neuroendocrine indices that demonstrate relatively graded responses to increasing intensities of stress, levels of pituitary cyclic AMP and plasma prolactin [11]. These two responses appear to be independently regulated, and therefore provide two relatively separate assessments of the degree of stress response. Pituitary cyclic AMP levels reflect stress-induced release of hypothalamic corticotropin releasing factor [9, 13–15], while prolactin release is affected primarily by changes in dopamine, endogenous opiates and serotonin [20].

We first replicated our original finding at a dose of 60 mg/kg of atropine sulfate using footshock as a stressor We then assessed the role of pain threshold in the observed effect of atropine. We next performed a dose response experiment in which lower doses of atropine sulfate were used prior to footshock. Finally, using the lowest and most pharmacologically relevant dose in terms of organophosphate antidote use, we tested and compared the effects of atropine sulfate and methylatropine nitrate (a quaternary compound

[1]Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23 All procedures were reviewed and approved by the WRAIR Animal Use Review Committee

[2]The views of the authors(s) do not purport to reflect the position of the Department of the Army or the Department of Defense (para 4-3, AR 360-5)

TABLE 1

EFFECTS OF ATROPINE PRETREATMENT ON RESPONSE TO FOOTSHOCK

Pretreatment	Control	Footshock
	Pituitary Cyclic AMP (pmoles/mg wet weight)	
Saline	1 43 ± 0 09	2 13 ± 0 26*
Atropine Sulfate	2 19 ± 0 15†	12 2 ± 3 0*†
	Plasma Prolactin (ng/ml)	
Saline	39 ± 8	237 ± 54*
Atropine Sulfate	21 ± 4	578 ± 254*

Values represent the mean ± SEM N=6 Animals were pretreated with saline or atropine sulfate (60 mg/kg) 15 min prior to 15 min of footshock Following injection, controls were replaced in their home cages for 30 min

*Significantly different than non-shocked controls, $p<0$ 05, Student's t-test, one-tailed

†Significantly different than saline-injected

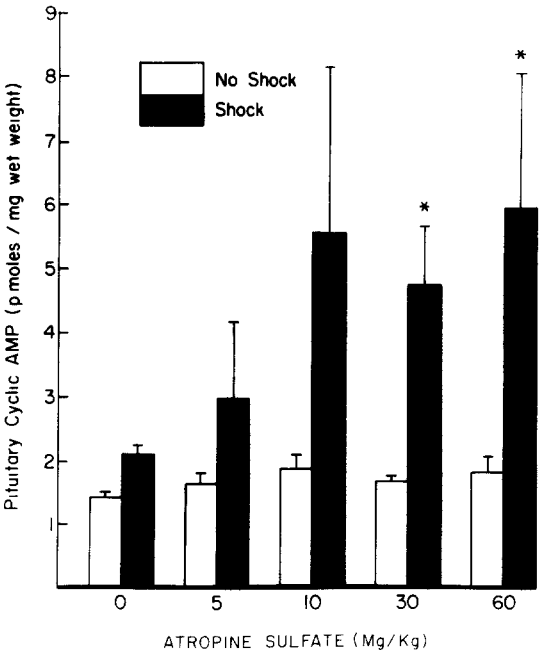

FIG 1 Effects of pretreatment with various doses of atropine administered 15 min prior to onset of 15 min intermittent footshock on levels of pituitary cyclic AMP Control animals were replaced in their home cages for 30 min following injection and then sacrificed *Significantly different from saline pretreated and shocked, $p<0$ 05, Student's t-test, one-tailed

that poorly penetrates the blood brain barrier) on pituitary cyclic AMP and plasma prolactin responses to immobilization stress

METHOD

Animals

Male Sprague-Dawley rats were purchased from Zivic-Miller and housed for a minimum of one week in our animal housing facility. Animals were housed in single hanging-wire cages with food and water freely available Lights were controlled on a 12 hr light-12 hr dark cycle (lights on 0700 to 1900 hr)

Drugs

Atropine sulfate and methylatropine nitrate were purchased from Sigma Chemical Co , St Louis, MO and prepared daily. Drugs were dissolved in saline and doses are expressed as the salt

Experimental Procedures

Footshock Animals were removed from their home cages and placed into a standard operant cage (33×33.5 cm equipped with parallel floorbars) housed inside an isolation box. The cages and boxes were purchased from BRS Foringer. Scrambled intermittent footshock was delivered to the floorbars on a variable interval schedule with an average intertrial interval of 30 sec. Footshock duration was 5 sec, footshock intensity was 1 6 mA The shockers and timing control equipment was purchased from Coulbourn Instruments. An average of 30 shock trials was delivered during the 15 min session.

Immobilization Rats were immobilized in 5.7 cm diameter plastic cylinders for 15 min prior to sacrifice. The plastic tube also served as the animal holder for use with the microwave device used to sacrifice animals in some experiments

Experiment 1 Effects of Atropine Sulfate (60 mg/kg) on Response to Footshock

Twelve rats were injected with saline and twelve rats were injected with atropine sulfate (60 mg/kg, IP) Control rats (unshocked, six rats from the saline and six rats from the atropine-injected group) were placed back into their cages for 30 min following injection and then sacrificed using microwave irradiation. Shocked rats (six saline pretreated and six atropine pretreated rats) were placed back into their home cages for 15 min following injection and then exposed to 15 min of intermittent footshock (described above) prior to sacrifice by microwave irradiation

Pituitary cyclic AMP and plasma prolactin were measured by radioimmunoassay (see below)

Experiment 2 Effect of Atropine Sulfate on Tail Flick Latency

Tail flick latencies in 12 rats were determined prior to injection Six rats were then injected with saline and six rats were injected with atropine sulfate (60 mg/kg) Latencies were measured again at 15 and 30 min post-injection At each time point, 3 trials utilizing 3 separate tail areas were averaged to calculate the latency

Experiment 3 Footshock Response Following Various Doses of Atropine Sulfate

Rats were pretreated with 0 (saline), 5, 10, 30 or 60 mg/kg atropine sulfate (IP) Twelve rats were injected with each dose Control rats (six rats from each dose group) were placed back into their home cages for 30 min prior to sacrifice Shocked rats (six from each dose group) were replaced into their home cages for 15 min and then exposed to 15 min of intermittent footshock immediately prior to sacrifice Rats were sacrificed by microwave irradiation Pitui-

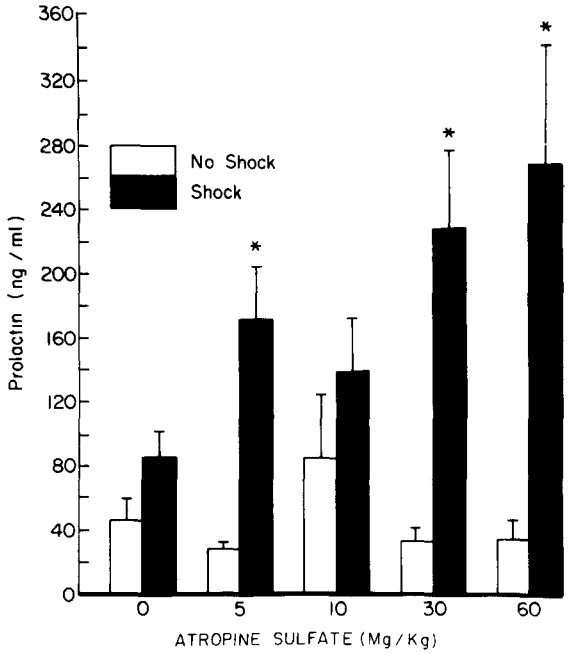

FIG 2 Effects of pretreatment with various doses of atropine administered 15 min prior to onset of 15 min intermittent footshock on levels of plasma prolactin Control animals were replaced in their home cages for 30 min following injection and then sacrificed *Significantly different from saline pretreated and shocked, $p<0$ 05, Student's t-test, one-tailed

tary cyclic AMP and plasma prolactin were measured by radioimmunoassay

Experiment 4 Comparision of Atropine Sulfate and Methylatropine Nitrate

Rats were pretreated with 5 mg/kg atropine sulfate or 5 mg/kg methylatropine nitrate or saline. Twelve rats were injected with each drug Control rats were placed back into their home cages for 30 min and then sacrificed. Immobilized rats were placed back into their home cages for 15 min and then restrained in plastic cylinders for 15 min prior to sacrifice. Rats were sacrificed by decapitation in this experiment because animal housing was no longer available near to the microwave system In stress studies, movement of rats from the housing area to the microwave could in itself be a stressor Therefore, animals were sacrificed by decapitation and the pituitary tissue was quickly heated as described below to avoid post-mortem changes in levels of pituitary cyclic AMP. Under these conditions, we have found that levels of pituitary cyclic AMP and plasma prolactin are equivalent following either decapitation or microwave sacrifice [11]

Sacrifice

Animals were either sacrificed by decapitation or by high power microwave irradiation (2450 mHz, 5 sec, 2 5 kW) depending upon the experiment. Pituitaries were dissected and weighed Pituitaries from animals sacrificed by decapitation were placed in 90°C sodium acetate buffer (pH 6.2, 0.05 M) to inactivate pituitary enzymes and minimize post-mortem changes in cyclic AMP Since microwave irradiation inactivates enzymes *in situ* [6,19], pituitaries from microwaved animals were placed in cold buffer. Following sonication and centrifugation, supernatants were stored at −70°C until assayed for cyclic AMP by radioimmunoassay Trunk blood was collected in heparinized beakers and plasma was stored at −40°C until assayed for prolactin

Assays

Cyclic AMP was determined by radioimmunoassay using antibodies produced in rabbits in our laboratory [18]. A highly specific antiserum was used at a final dilution of 1 400,000 The antiserum exhibited cross-reactivities for ATP and cyclic GMP of less than 0.00007 and 0.14% respectively Within assay variation was 7% and between assay variation was 18%

Materials for the prolactin assay were provided by the National Institute of Health through the Rat Pituitary Hormone Distribution Program. Prolactin was radioiodinated as previously described [18]. Within assay variation was 8% and between assay variation 12%

Statistics

Planned comparisons were made using Student's t-test. A one-tailed test was used since these experiments were performed to test the specific hypotheses that atropine increased responses to stress and decreased pain threshhold

RESULTS

As shown in Table 1, footshock stress increased levels of pituitary cyclic AMP and plasma prolactin Atropine sulfate pretreatment (60 mg/kg) increased these responses as compared to saline-pretreated rats Atropine sulfate also significantly increased levels of pituitary cyclic AMP in the nonshocked rats However, this effect was not replicated in the dose-response experiment described below The effect of atropine pretreatment plus stress was much greater than the additive effects of atropine and stress given separately

Atropine-pretreated rats exhibited a significantly decreased latency between application of heat source and tail flick as compared to saline-pretreated rats. Fifteen minutes following injection, tail flick latencies for atropine sulfate pretreated rats averaged (mean±SEM) 4 04±0.39 sec as compared to saline pretreated rats, 5.75±0.26 sec. This difference was statistically significant, $p<0$ 05, Student's t-test, $t=3.5$, one-tailed The latencies of the atropine-pretreated group at 30 min post-injection averaged 5 30±0.58 as compared to 6 20±0.38 for the saline injected rats, but this difference was not statistically significant

Potentiation of stress response by atropine sulfate was not limited to the 60 mg/kg dose as seen in Figs. 1 and 2 Rats pretreated with 5, 10, 30 or 60 mg/kg atropine sulfate showed an increased pituitary cyclic AMP response to footshock with statistically significant differences observed between the 30 or 60 mg/kg pretreated groups vs saline-pretreated rats. Prolactin response to footshock was also increased in all atropine-pretreated groups with statistically significant differences observed between the 5, 30 and 60 mg/kg groups vs saline-pretreated rats

This potentiated stress response following atropine pretreatment was not limited to footshock stress as seen in Table 2 Neither atropine sulfate nor methyl atropine nitrate significantly increased non-stressed levels of pituitary cyclic AMP or plasma prolactin Atropine sulfate (5 mg/kg) but not

TABLE 2
EFFECTS OF ATROPINE AND METHYLATROPINE ON RESPONSE TO IMMOBILIZATION

Pretreatment	Control	Immobilization
	Pituitary Cyclic AMP (pmoles/mg wet weight)	
Saline	0.8 ± 0 2	2.9 ± 0.9*
Atropine Sulfate	1.2 ± 0 5	13 4 ± 4.6*†
Methylatropine Nitrate	1.0 ± 0.2	1 9 ± 0 4*
	Plasma Prolactin (ng/ml)	
Saline	14 ± 4	86 ± 17*
Atropine Sulfate	13 ± 3	100 ± 21*
Methylatropine Nitrate	15 ± 2	74 ± 11*

Values represent the mean ± SEM N=6. Animals were pretreated with saline, methylatropine nitrate (5 mg/kg) or atropine sulfate (5 mg/kg) 15 min prior to 15 min of immobilization. Following injection, controls were replaced in their home cages for 30 min

*Significantly different than control, $p<0$ 05, Student's t-test, one-tailed

†Significantly different than saline-injected, $p<0.05$, Student's t-test

methylatropine nitrate (5 mg/kg) increased pituitary cyclic AMP response to immobilization. Immobilization significantly increased levels of plasma prolactin, but neither atropine sulfate nor methylatropine nitrate potentiated prolactin response in this experiment.

DISCUSSION

The experiments presented in this report demonstrate that atropine sulfate potentiates pituitary cyclic AMP and plasma prolactin responses to stress. Atropine's effect could be due to three different types of mechanisms or to a combination of them. Atropine could directly affect biochemical regulation of the pituitary gland, either by direct blockade of cholinergic pituitary receptors or via effects on cholinergic neurons involved in neuroendocrine regulation. A third possibility is that atropine affects the perception of the stress intensity. These possibilities will be considered in turn.

Although cholinergic muscarinic receptors are located in the pituitary gland [4, 22, 27], we feel it is unlikely that the observed potentiation of pituitary cyclic AMP stress responses by atropine occurs at the pituitary level. Since the pituitary gland lies outside the blood brain barrier, methylatropine nitrate would have been effective if the effects of atropine were mediated via direct blockade of pituitary muscarinic receptors. The failure of methylatropine nitrate, a peripherally acting atropine compound that poorly penetrates the blood brain barrier [29], to mimic the effects of atropine sulfate suggests that the relevant site for this action of atropine is central rather than peripheral. Since neither atropine sulfate nor methylatropine nitrate affected prolactin response to immobilization at the low 5 mg/kg dose used in this experiment, the site of the effects of atropine on the prolactin response in the footshock experiments cannot be determined from these data.

Hypothalamic cholinergic neurons are involved in neuroendocrine regulation, and the effects of atropine might be mediated at pre or post-synaptic receptors of these cholinergic neurons. We have shown that stress-induced increases in pituitary cyclic AMP are primarily the result of stress-induced release of corticotropin releasing factor (CRF) from the hypothalamus and the subsequent stimulation of pituitary CRF receptors linked to adenylate cyclase [13] The increased synthesis of pituitary cyclic AMP is related to the stress-induced release of pituitary hormones regulated by CRF, i.e , ACTH, β-endorphin and β-lipotrophic hormone [9]. However, cholinergic neurons appear to be stimulatory for CRF release [7], and therefore cholinergic agonists rather than antagonists would be predicted to increase CRF release and thereby increase levels of pituitary cyclic AMP. In fact, administration of cholinergic agonists such as the muscarinic agonist, oxotremorine, increases levels of pituitary cyclic AMP [17,21] Thus, it seems improbable that direct pharmacological actions of atropine at hypothalamic cholinergic sites directly involved in CRF regulation are the cause of the observed potentiation of stress responses by atropine pretreatment. However, atropine might act at other central cholinergic pathways.

Cholinergic neurons also appear to be involved in pain perception and reactivity to stimuli. Depletion of acetylcholine by electrolytic lesion or neurotoxin administration has been reported to increase reactivity to handling [5,25] Scopolamine (a muscarinic antagonist) decreases the amount of footshock required to elicit escape behavior [3]. Cholinergic agonists or cholinesterase inhibitors have been shown to increase pain threshholds [24,26]. In the present report, atropine (60 mg/kg) decreased tail flick latencies These data and reports are consistent with the hypothesis that cholinergic agonists tend to increase pain thresholds, while cholinergic antagonists decrease pain thresholds The potentiation of stress responses that we observed might thus be due, in part, to decreased pain thresholds. However, we also tested a stressor that did not involve pain (immobilization). Yet, the pituitary cyclic AMP response to this stressor was also potentiated by atropine pretreatment. Therefore, lowering of pain threshold is probably not the sole mechanism of action for atropine's effect in potentiating stress response.

Recently, it has been reported that atropine pretreatment potentiated corticosterone response to immobilization, cold exposure or footshock of 1 to 4 hr duration [1,23]. Atropine also has been reported to potentiate plasma glucose responses to some stressors [1]. In addition high doses of atropine (>80 mg/kg) combined with cold (16°C) swim stress produced convulsions and death in male mice [2].

Thus, while the exact mechanism has not been determined, the experiments described in this report and others demonstrate that the response to stress may be influenced by pretreatment with atropine and conversely that the response to atropine may be influenced by stress

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