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Biochemical Pharmacology, Vol. 30, No. 24, pp. 3377–3380, 1981.
Printed in Great Britain.

0006-2952/81/243377-04 $02.00/0
Pergamon Press Ltd.

Increases in cyclic AMP levels in rat brain regions *in vivo* following isoproterenol*

(*Received* 5 *March* 1981; *accepted* 12 *June* 1981)

Cyclic AMP may serve as a second messenger within the CNS [1, 2]. It is distributed throughout rat brain and responds to pharmacological and physiological manipulations [3–7]. The physiological effects of beta-adrenergic stimulation by cathecholamines in the periphery are thought to be mediated by adenyl cyclase activation [8]. Beta-adrenergic receptors have been described and measured by radioligand techniques throughout the mammalian CNS [9, 10]. Cyclic AMP increases in tissue from various brain regions after *in vitro* incubation with beta-adrenergic agonists such as isoproterenol suggesting that cyclic AMP may mediate the effect of beta-adrenergic stimulation in the CNS [11–13]. We were interested in determining whether *in vivo* administration of isoproterenol would alter cyclic AMP concentrations in selected regions of the brain.

The pineal has a beta-adrenergic receptor whose noradrenergic input from the superior cervical sympathetic ganglion varies greatly during the day versus the night [14, 15]. The sensitivity of the pineal beta-receptor also varies dramatically during the normal 24-hr period. The greatest sensitivity is seen during the light hours, and pineals of animals maintained in constant light show a supersensitive response *in vitro* to norepinephrine [16]. Therefore, in a separate experiment we studied cyclic AMP responsiveness to isoproterenol *in vivo* in animals maintained under constant light conditions.

Methods

Animals. In the first experiment, male Sprague–Dawley albino rats† (250–350 g) were obtained from Taconic Farms. In the second experiment, Wistar-derived rats from the Walter Reed Army Institute of Research colony were used. The animals had free access to food and water and were maintained in a 12 hr light–dark cycled room with lights on from 6.00 a.m. to 6.00 p.m. except for one group of rats in the second experiment which were maintained in constant light for 3 weeks. Experiments were performed between 8:30 a.m. and 12:30 p.m. to minimize circadian effects.

Solutions. DL-Isoproterenol HCl (Sigma) was dissolved in saline. The dose is expressed as the salt.

Habituation. The animals were habituated to the experimental procedure to minimize stress effects. Rats were injected with saline for a minimum of 3 days prior to the experimental day. Ten minutes after the injection, the rats were habituated to passing through a plastic open-ended cylinder similar to the plastic microwave applicator to be used on the experimental day. The animals were then returned to their home cage. Animals used in experiment 1 were habituated for 3 days, while rats in experiment 2 were habituated for 10 days. The longer habituation resulted in lower variability of cyclic AMP levels in saline-injected animals (see Discussion).

Experimental procedures. In the first experiment rats were alternately injected (i.p.) with either saline, 10 mg/kg isoproterenol, 20 mg/kg isoproterenol, or 30 mg/kg isoproterenol. Ten minutes later the rats were placed in a plastic

* This material has been reviewed by the Walter Reed Army Institute of Research, and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

† In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", a promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

microwave applicator cylinder similar to the tube the rats had passed through during the habituation procedure. In this case, however, the cylinder was not open-ended. An immobilizing plunger was quickly inserted behind the rat, and the applicator tube was inserted into a microwave wave guide. The rats were killed within 30 sec from the time the immobilizing plunger was inserted.

In the second experiment one group of rats had been maintained in constant light for 3 weeks and the other group maintained on a 12 hr light–dark cycle. All rats were killed in the light, approximately 3–6 hr after "lights on" for the light–dark cycled group. Rats in each group were injected with saline or 10 mg/kg isoproterenol and otherwise treated as in experiment 1.

The lowest dose of isoproterenol was chosen since a supersensitive response was expected.

Microwave fixation and sample preparation. The rats were placed in the plastic applicator tube which was then inserted into a hole in the short-circuiting endplate of a WRC 430 wave guide exposure chamber in such a manner that the longitudinal axis of the rat head was perpendicular to the microwave E field [17, 18]. The rats were irradiated at 2450 MHz for 5 sec using 2.5 kW forward power. Following microwave irradiation, the heads were cooled on dry ice and then the brain regions were dissected as previously described [19]. The tissue pieces were weighed, placed in 50 mM sodium acetate buffer (pH 6.2) and sonicated with a Heat System model 185 sonicator for 5–30 sec depending on tissue size at a power setting of 50 W. The sonicates were centrifuged at 25,000 *g* for 15 min and then the supernatant fractions were stored at −70° until assayed.

Cyclic nucleotide assay. Cyclic AMP was determined by a modification of the radioimmunoassay described by Steiner *et al.* using antibodies characterized in our laboratory [3,20]. For measurement of the cyclic nucleotides in the smaller brain regions, a further modification of the method described by Harper and Brooker [21] was employed.

The data were analyzed by computer using a non-linear four parameter logistic model weighted for non-uniformity of variance [22]. The minimal detectable amount of cyclic AMP was 0.10 pmole/assay tube for the regular assay and 3 fmoles for the acetylated assay. Phosphodiesterase treatment of tissue extracts reduced cyclic AMP to undetectable levels.

Results

Experiment 1. As shown in Table 1, isoproterenol increased levels of cyclic AMP in all regions examined. In this experiment the levels were increased in nineteen of the twenty-one regions at a dose of 10 mg/kg, in twenty out of twenty-one at 20 mg/kg, and in all regions at 30 mg/kg. Generally the increases were not statistically significant at 10 or 20 mg/kg but most increases were significant at 30 mg/kg. The largest increase after 30 mg/kg was seen in the pineal (10-fold) although no increase was seen after the 10 or 20 mg/kg doses. The pituitary cyclic AMP tripled after 30 mg/kg and increases in most other regions ranged between 30 and 60 per cent at this dose. The midbrain and nucleus accumbens showed the least increase.

Experiment 2. Isoproterenol (10 mg/kg) increased levels of cyclic AMP in all ten regions examined in the light–dark cycled rats (Figs. 1 and 2). The pineal and pituitary were most responsive. In the rats maintained in constant light, the cyclic AMP response to isoproterenol was increased in the pineal (Fig. 1) but not increased in other regions examined (Fig. 2). In six regions no significant increase in cyclic AMP was seen in the constant light animals although significant increases were seen in the light–dark group.

Discussion

These experiments demonstrate that isoproterenol stimulates cyclic AMP production *in vivo* throughout the rat brain and the pineal and pituitary glands, presumably by stimulation of beta-adrenergic receptors linked to adenylate cyclase. These data confirm and extend the findings of Nahorski *et al.* [23–25] who demonstrated *in vivo* that isoproterenol increases cyclic AMP in the forebrain of young chicks with an immature blood brain barrier.

Cyclic AMP levels were increased significantly in nineteen of the twenty-one regions examined at the 30 mg/kg dose. This widespread cyclic AMP response was not unex-

Table 1. Effects of isoproterenol on cyclic AMP *in vivo**

Region	Cyclic AMP (pmoles/mg wet weight ± S.E.M.) Saline	Isoproterenol (10 mg/kg)	Isoproterenol (20 mg/kg)	Isoproterenol (30 mg/kg)
Pituitary	1.060 ± 0.162	1.205 ± 0.257	1.899 ± 0.543	3.070 ± 0.482†
Pineal	2.558 ± 1.324	2.049 ± 0.326	2.780 ± 0.488	24.51 ± 10.60†
Cerebellum	0.650 ± 0.068	0.755 ± 0.128	0.744 ± 0.080	0.833 ± 0.108†
Brainstem	0.422 ± 0.049	0.497 ± 0.047	0.488 ± 0.044	0.726 ± 0.058†
Midbrain	1.026 ± 0.047	1.174 ± 0.090	1.186 ± 0.196	1.128 ± 0.097
S. nigra	0.719 ± 0.112	1.022 ± 0.045†	0.955 ± 0.092	1.329 ± 0.097†
Interpeduncular n.	0.824 ± 0.129	0.877 ± 0.113	1.093 ± 0.221	1.743 ± 0.805
S. colliculus	1.077 ± 0.052	1.234 ± 0.056	1.478 ± 0.316	1.724 ± 0.236†
I. colliculus	1.034 ± 0.047	1.160 ± 0.072	1.425 ± 0.330	1.546 ± 0.180†
Hippocampus	0.656 ± 0.048	0.675 ± 0.046	0.763 ± 0.044	1.017 ± 0.185†
Amygdala	1.220 ± 0.089	1.413 ± 0.227	1.551 ± 0.264	2.679 ± 0.763†
Pyriform cortex	1.453 ± 0.146	1.458 ± 0.209	1.568 ± 0.119	1.905 ± 0.158†
Thalamus	0.777 ± 0.045	0.846 ± 0.052	0.906 ± 0.035†	0.993 ± 0.077†
Hypothalamus	0.646 ± 0.075	0.715 ± 0.047	0.738 ± 0.113	1.047 ± 0.071†
Striatum	0.636 ± 0.017	0.815 ± 0.058†	0.745 ± 0.048†	0.820 ± 0.064†
Septal region	1.062 ± 0.066	1.493 ± 0.263	1.251 ± 0.126	1.676 ± 0.228†
N. accumbens	0.951 ± 0.044	0.939 ± 0.084	0.917 ± 0.042	1.121 ± 0.078†
O. tubercle	1.009 ± 0.090	0.981 ± 0.091	1.079 ± 0.159	1.291 ± 0.086†
Cortex	0.593 ± 0.043	0.616 ± 0.070	0.666 ± 0.088	0.862 ± 0.026†
Frontal cortex	0.753 ± 0.102	1.189 ± 0.114	0.954 ± 0.089	1.228 ± 0.142†
O. bulb	1.042 ± 0.114	1.155 ± 0.120	1.059 ± 0.099	1.337 ± 0.067†

* Rats were injected (i.p.) with isoproterenol or saline as indicated, and then killed 10 min later by microwave irradiation. N = 6 per group.

† Significantly different ($P < 0.05$) from saline-injected animals (Student's *t*-test).

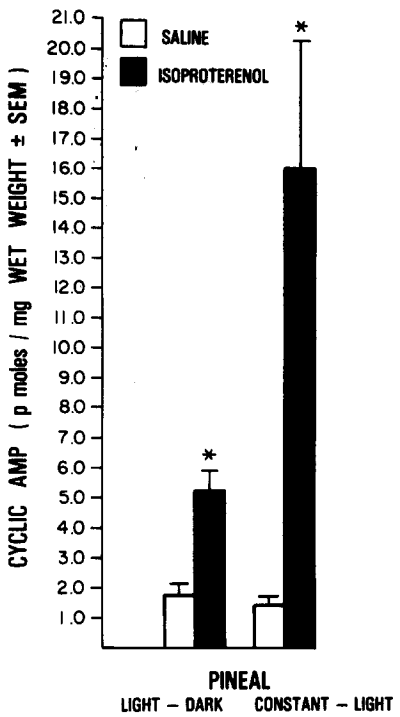

Fig. 1. Pineal cyclic AMP response to isoproterenol. Rats were maintained either in constant light or under a 12 hr light–dark cycle for 3 weeks prior to being killed. Rats were injected (i.p.) with saline or isoproterenol (10 mg/kg), and then killed 10 min later by microwave irradiation. N = 6 per group. Key: (*) Significantly different ($P < 0.05$) from saline-injected animals (Student's *t*-test).

pected since norepinephrine, beta-adrenergic receptors, and isoproterenol-sensitive adenylate cyclase have been found throughout the rat brain [26–30]. However, there were no apparent correlations between the magnitude of the cyclic AMP response observed in a particular region and the reported density of beta-receptors for that region. For example, the striatum and cortex are reported to have approximately twice the density of beta-receptors as the cerebellum and hypothalamus [28, 30], yet the increase in cyclic AMP seen after 30 mg/kg isoproterenol was similar in these regions. Also, no apparent differences in beta-receptor subtype cyclic AMP response to isoproterenol were seen. The cortex, containing primarily beta-1 receptors, and the cerebellum, containing primarily beta-2 receptors [30], responded similarly.

The largest cyclic AMP responses were seen in the pituitary and pineal which lie outside the blood–brain barrier. Only a small percentage of the isoproterenol is taken up by the brain from the circulation [31]. Therefore the effective isoproterenol concentration outside the blood–brain barrier was much greater than inside the barrier. It is possible that some of the observed cyclic AMP response resulted from stimulation of beta-adrenergic receptors located in the cerebral capillaries [32], or was caused indirectly from isoproterenol-induced peripheral effects.

The sensitivity of the pineal receptor–cyclase complex has been shown to vary with the light–dark cycle, being least sensitive at night when the norepinephrine input is greatest and most sensitive during the light hours when the sympathetic innervation is relatively quiescent. This has been demonstrated *in vitro* and *in vivo* [16, 33, 34]. We found a supersensitive pineal cyclic AMP response to isoproterenol in animals kept in constant light. The saline-injected rats, however, had similar cyclic AMP levels in the pineal in both lighting regimens. The underlying supersensitivity was only revealed when a challenge was administered.

A second interesting finding was that the cyclic AMP increase after isoproterenol appeared to be attenuated in most regions in the constant light group compared to the light–dark cycled group. A recent study by Wirz-Justice *et al.* [35] reports that the number of alpha- and beta-adrenergic receptors in rat forebrain undergo circadian rhythms. From this report, one would expect differential cyclic AMP response to a given dose of isoproterenol at different times of the day, depending on the number of receptors present. The effect of constant light on beta-receptor rhythms in regions other than the pineal is not known.

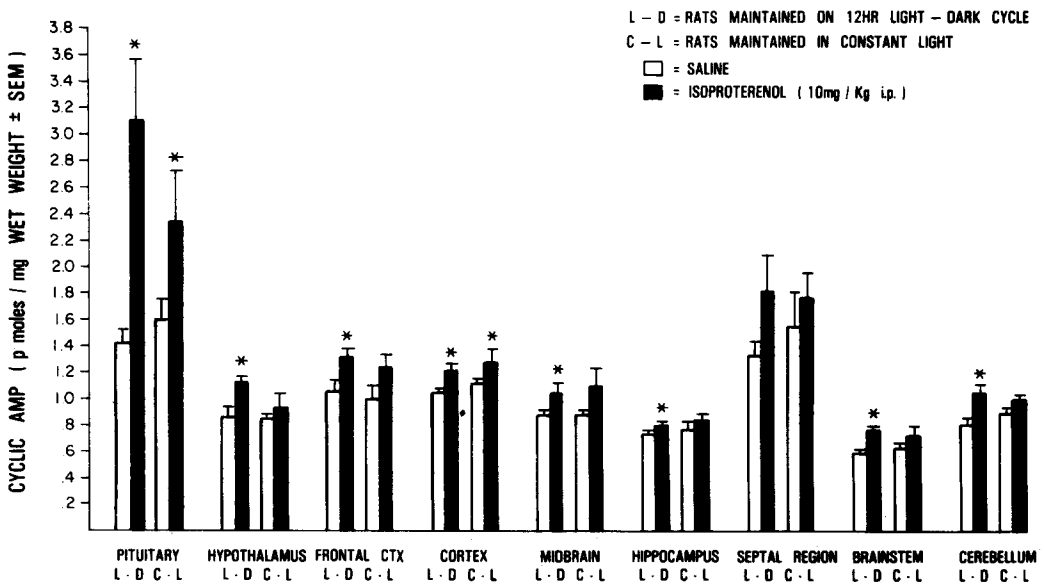

Fig. 2. Brain and pituitary cyclic AMP response to isoproterenol. See Fig. 1 legend for experimental protocol. N = 6 per group. Key: (*) Significantly different ($P < 0.05$) from saline-injected animals (Student's *t*-test).

In comparing the data from the two experiments, it can be seen that 10 mg/kg of isoproterenol significantly increased levels of cyclic AMP in the second experiment, while a higher dose (30 mg/kg) was required to achieve statistically significant increases in the first experiment. This may be due to relative stress effects in the two experiments. Stress has been shown to release norepinephrine [36, 37] and also possibly to increase cyclic AMP according to a recent report [38]. The rats in the second experiment were habituated to the experimental procedure for a longer period of time (10 vs 3 days) prior to the experiment. This is reflected in the lower variability associated with the saline-injected cyclic AMP levels. Possibly the decreased "stress" effect enables the isoproterenol effect to be evident at a lower dose.

In summary, the major finding of this report is that stimulation of CNS beta-receptors *in vivo* with isoproterenol results in increased levels of cyclic AMP, thus suggesting that, as in the periphery, cyclic AMP serves as a second messenger in mediating beta-adrenergic receptors.

Acknowledgements—The authors wish to thank Clyde Kenion, Jr., Golden Driver, Rosa Thorpe, David Collins, and Clinton Wormley for their expert technical assistance and Pat Conners for typing the manuscript.

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