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Effect of (1–24)Adrenocorticotrophin on the Uptake of ^{3}H-Norepinephrine by the Rat Atria

P. A. MEHRABANI AND J. R. BASSETT[1]

School of Biological Sciences, Macquarie University, N. S. W. 2109, Australia

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MEHRABANI, P. A. AND J. R. BASSETT. *Effect of (1–24)adrenocorticotrophin on the uptake of ^{3}H-norepinephrine by the rat atria.* PHARMACOL BIOCHEM BEHAV **30**(2) 391–396, 1988.—Spontaneously beating rat atria were incubated with ^{3}H-norepinephrine both in the presence and absence of (1–24)ACTH. A significant reduction in the uptake and retention of radioactivity was found in atria pretreated with (1–24)ACTH. A kinetic study of the uptake process showed similar K_m values for both the control (24.1×10^{-8} M) and (1–24)ACTH pretreated (22.2×10^{-8} M) groups, but a significantly different V_{max}. The K_m values were similar to that reported for the neuronal reuptake process (Uptake 1). It was concluded that the ACTH-induced enhanced myocardial sensitivity to catecholamines previously reported, could be explained in part on the basis of an inhibition of neuronal uptake by (1–24)ACTH. The inhibition of neuronal uptake by (1–24)ACTH was dose-dependent.

ACTH Norepinephrine Neuronal uptake Uptake inhibition Myocardial sensitivity

BASSETT and Cairncross [3] reported that the exposure of male rats to irregular signalled footshock resulted in an enhanced myocardial sensitivity to both norepinephrine and epinephrine. Such an enhanced sensitivity could be explained in part on the basis of an inhibition of neuronal uptake. Spontaneously beating atria from rats previously exposed to footshock stress were found to exhibit a significant reduction in the uptake and retention of ^{3}H-norepinephrine [4]. A kinetic study of the uptake process in both stressed and control groups suggested that it was the neuronal uptake of catecholamines that was inhibited. While it was originally suggested that the glucocorticoids may be involved in the inhibition of uptake, and the resulting enhanced myocardial sensitivity to catecholamines, Bassett, Strand and Cairncross [5] found that adrenocorticotropic hormone (ACTH) was more potent than the glucocorticoids in inducing such an enhanced sensitivity. While ACTH and related peptide fragments have been shown to have a direct action on central nervous system structures, resulting in modification of adaptive behaviour such as avoidance, approach, motivation, and even aggression and sexual response [6,26], ACTH has not previously been associated with catecholamine uptake processes and no receptors for ACTH have so far been demonstrated in cardiac tissue. The present study was undertaken to see if (1–24) ACTH would affect the uptake and retention of ^{3}H-norepinephrine into cardiac tissue, and so explain its action in enhancing myocardial sensitivity to the catecholamines.

METHOD

Animals

Male CSF rats, 90±5 days old were used in this study. The animals were housed in groups of 3 under conditions of constant temperature and humidity (21±0.5°C, 46% humidity) and subjected to a 12-hr reversed night-day schedule (light 2000 to 0800 hr) beginning at least 14 days prior to the commencement of experimentation. Food and water were provided ad lib.

Tissue Preparation

The methods are similar to those reported by Bassett and Cairncross [4]. The atria were suspended in an organ bath, containing 10 ml Krebs-Henseleit solution gassed with 5 percent carbon dioxide in oxygen and maintained at 29°C. The Krebs solution had the following composition (mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25.0; $MgSO_4$ 0.45; KH_2PO_4 1.03; $CaCl_2$ 2.5; D(+) glucose 11.1. Atropine sulphate at a concentration of 2.9×10^{-8} M was added to the Krebs solution. The inotropic response of the spontaneously beating atria was monitored using an isotonic strain gauge transducer. Preparations were allowed to equilibrate for 30 min after setting up, before commencement of the experiment. Ethylenediamine tetra acetic acid (EDTA), 20 μg/ml was added to the solution in the organ bath 5 min before the addition of norepinephrine. Cumulative log dose-response curves for norepinephrine were obtained in triplicate. Responses were ex-

[1]Requests for reprints should be addressed to Dr. J. R. Bassett.

pressed as a percentage of the maximal response determined for each curve. Regression lines were fitted to the linear portions of the curves by the method of least squares. Each line was tested for linearity and the ED_{50} calculated using regression coefficients. The ED_{50}'s from 7 experiments were measured and the mean ED_{50} calculated. This concentration of norepinephrine was used in the subsequent experiments.

Accumulation of Radioactivity as a Function of Time

Spontaneously beating atria were incubated with 1-[7,8-^{3}H] norepinephrine (2.79×10^{-7} M) for periods of 5, 7, 10, 15 and 20 min. The ^{3}H-norepinephrine (specific activity 41.4 Ci/mmole) was obtained chromatographically pure from Amersham International Ltd. Following incubation with the labelled amine, the atria were removed from the organ bath, washed in Krebs-Henseleit solution and blotted, this procedure being repeated 3 times. The tissues were then weighed and homogenized in 3 ml of 0.4 M perchloric acid containing 10 μg/ml cold norepinephrine. The homogenate was centrifuged at 1500×g for 15 min, then duplicate 500 μl aliquot of the supernatant were taken and added to 10 ml of scintillation fluid (Pico-Fluor 30; United Technologies Packard) and counted in a Packard scintillation counter. The automatic external standardization ratio technique was used to monitor quenching. Efficiency curves were obtained and all counts were corrected for counting efficiency to give the number of disintegrations/min (DPM). Total tissue radioactivity was then calculated as DPM/mg of atrial tissue. The accumulation of radioactivity in atria from both ACTH pretreated and control animals after various incubation periods was compared using unpaired t-tests.

In the case of tissues pretreatment with ACTH, (1–24)ACTH (Synacthen; Ciba-Geigy) at a nominal bath concentration of 8.5×10^{-9} M was added to the organ bath 10 min before the addition of ^{3}H-norepinephrine. The incubation time was the same as used by Bassett, Strand and Cairncross [5] and the concentration was that reported to produce the maximum potentiation of myocardial sensitivity to norepinephrine [5].

Determination of Incubation Time for Kinetic Analysis

The uptake of ^{3}H-norepinephrine (in DPM/mg) with time was measured using both the largest (8.28×10^{-7} M) and smallest (1.04×10^{-7} M) concentrations of norepinephrine to be used in the kinetic analysis of the uptake process. From this study a suitable incubation time, lying on the linear portion of both uptake curves, was chosen. In this experiment atria that had been washed, blotted and weighed were digested in 1.0 ml of HCS tissue solubilizer (Amersham) instead of homogenisation. After digestion partial neutralization for sample clarity was achieved by adding 30 μl of glacial acetic acid before the addition of 10 ml of scintillation fluid. This digestion method was found to reduce the variability in the results and was used in all subsequent experiments.

Initial Rate of Norepinephrine Uptake at Various Incubation Concentrations

The method was essentially the same as that described above, except that in this series of experiments the incubation time was fixed at 7 min and the concentration of ^{3}H-norepinephrine was varied. The concentrations of norepinephrine used were 1.04, 2.07, 4.14, 6.21 and 8.28×10^{-7} M. The number of animals per group is shown in Table 2. At the higher concentrations of norepinephrine unlabelled l-norepinephrine was added to the bath to make up the required concentrations. The DPM'S measured were corrected accordingly. The initial rate of norepinephrine uptake (v) was calculated as DPM/mg/min and the results expressed as a Michaelis-Menten plot [15].

The initial rate of norepinephrine uptake at the various concentrations of norepinephrine was plotted in the form of S/v against S, where S=molar bath concentration of norepinephrine. From the slopes and intercepts of the graphs the kinetic constants V_{max} (maximum rate of uptake) and K_m (Michaelis-Menten constant) were determined [15].

The Effect of Various Doses of (1–24)ACTH on the Uptake of Norepinephrine

All doses of (1–24)ACTH were added to the organ bath 10 min before the addition of ^{3}H-norepinephrine (2.79×10^{-7} M). The incubation time following the addition of norepinephrine was 7 min. The nominal bath concentrations of (1–24)ACTH used in this experiment were 0.85, 8.5, 85 and 850×10^{-9} M. In all cases the volume of ACTH solution added to the organ bath was the same (100 μl).

Measurement of the Actual Bath Concentration of (1–24)ACTH

While (1–24)ACTH was added to the organ bath to give the nominal concentrations stated above, it must be remembered that adsorption of ACTH to the glass walls of the bath will be a major source of loss of the peptide [8]. Adsorption losses, together with the rapid destruction of the ACTH molecule by enzymes released by degenerating cells during the incubation period [8], will result in a considerable loss of both biological and immunological activity.

In order to more accurately measure the level of (1–24)ACTH available to the tissue receptors, the concentration of (1–24)ACTH in the organ bath was assayed. The organ bath was set up as before with 10 ml Krebs-Henseleit solution gassed with 5 percent carbon dioxide in oxygen and maintained at 29°C. Atropine sulphate and EDTA were added to the bath as described previously. ACTH was then added to the organ bath to give nominal bath concentrations of either 8.5 or 85×10^{-9} M (the two concentrations found to significantly effect the uptake of ^{3}H-norepinephrine). Samples were removed from the organ bath at 1, 5 and 10 min after the addition of the (1–24)ACTH and immediately snap frozen in liquid nitrogen. The samples were then stored at −20°C in plastic containers until analysis. This experiment was repeated at least six times for each nominal bath concentration.

Analysis of (1–24)ACTH

The actual concentration of (1–24)ACTH in the organ bath was analysed by the radioimmunoassay method described in detail by Donald [8]. Briefly the method involves the extraction of ACTH using silicic acid powder and its subsequent removal from the silicic acid with an acetone/water/glacial acetic acid (100:25:1) mixture. The pH of the extract was corrected to between 7 to 8 units by the addition of ammonia solution, and the protease inhibitor Trasylol (Bayer AG, Germany) added. Antiserum raised in rabbit against (1–24)ACTH was added and the mixture incubated for 3 days at 4°C. Purified ^{125}I-(1–24)ACTH was then added

TABLE 1

THE ACCUMULATION OF RADIOACTIVITY IN ISOLATED SPONTANEOUSLY BEATING RAT ATRIA FOLLOWING INCUBATION WITH L-[7,8-^{3}H] NOREPINEPHRINE IN THE PRESENCE AND ABSENCE OF A NOMINAL CONCENTRATION OF 8.5×10^{-9} M (1–24)ACTH

Incubation Time Min	Uptake of Radioactivity (DPM/mg) Mean ± S.E.M. Control	ACTH	*t*-test (*p*)
5	2640 ± 152 (6)	2172 ± 199 (6)	N.S.
7	4207 ± 186 (9)	3590 ± 58 (8)	<0.01
10	5759 ± 197 (8)	4352 ± 115 (6)	<0.001
15	7197 ± 265 (10)	6223 ± 276 (9)	<0.002
20	9992 ± 373 (5)	9924 ± 599 (5)	N.S.

Numbers in parentheses = Number of animals/group.
N.S. = Not significant ($p>0.05$; unpaired *t*-test).

TABLE 2

NUMBER OF ANIMALS PER GROUP USED IN THE MICHAELIS-MENTEN ANALYSIS OF THE INITIAL RATE OF UPTAKE OF ^{3}H-NOREPINEPHRINE

Bath Concentration of Norepinephrine ($\times 10^{-7}$ M)	Number of Animals Control	ACTH Pretreated
1.04	5	4
2.07	5	4
4.14	5	8
6.21	5	7
8.28	5	4

and the tubes incubated for a further 2 days at 4°C. Both the (1–24)ACTH antiserum and the ^{125}I-(1–24)ACTH were graciously provided by C.S.I.R.O. (Prospect, N.S.W.). Synthetic (1–24)ACTH (Synacthen, Ciba) was used to prepare the standard curve. Unbound ^{125}I-(1–24)ACTH was removed using an activated charcoal solution (500 mg charcoal, 50 ml deionised water and 5 ml of ACTH free plasma). The tubes were centrifuged at 4000 rpm for 15 min and an aliquot of the supernatant taken and counted in a gamma counter. The results were plotted as the ratio of bound/unbound hormone and the concentrations of the unknown solutions read from the standard curve. An internal reference solution of known (1–24)ACTH concentration (C.S.I.R.O., Prospect) was run with each assay to check the accuracy of the analysis.

RESULTS

The mean ED_{50} (±S.E.M.) for l-norepinephrine on spontaneously beating rat atria, determined using 7 animals, was $2.67 \pm 1.44 \times 10^{-7}$ M. Spontaneously beating atria were then incubated with 1-[7,8-^{3} H] norepinephrine at a dose approximating the ED_{50} (2.79×10^{-7} M) for various time periods. The accumulation of radioactivity in atria in the presence and absence (Control) of (1–24)ACTH (nominal concentration 8.5×10^{-9} M) is shown in Table 1. A significant reduction in the accumulation of radioactivity was observed

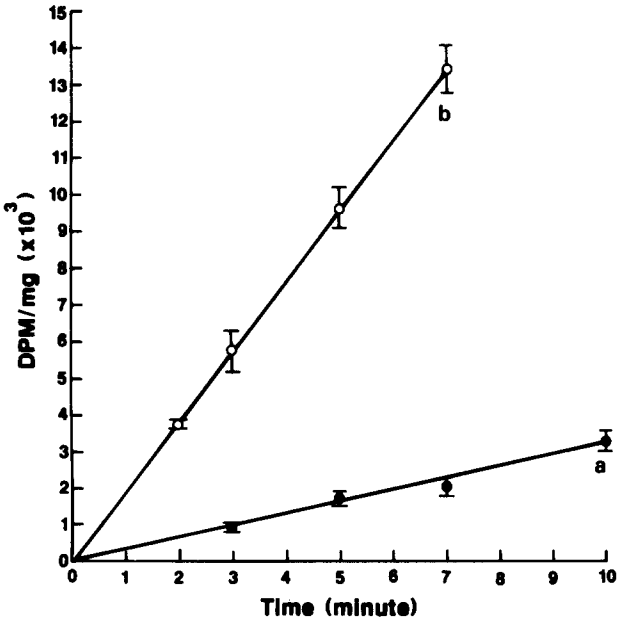

FIG. 1. The accumulation of radioactivity with time in isolated spontaneously beating atria following incubation with ^{3}H-norepinephrine at bath concentrations of 1.04×10^{-7} M (●) and 8.28×10^{-7} M (○). Each point represents the mean of 6 atria and the vertical bars denote ±S.E.M.

in the atria incubated in the presence of (1–24)ACTH following incubation times with ^{3}H-norepinephrine of 7, 10 and 15 min (unpaired *t*-tests; $p<0.05$ in all cases). No significant reduction was observed following incubation for 5 and 20 min.

The accumulation of radioactivity with time in isolated spontaneously beating atria following incubation with ^{3}H-norepinephrine at doses of 1.04 and 8.28×10^{-7} M are shown in Fig. 1. These two doses of ^{3}H-norepinephrine represent the upper and lower doses used in the kinetic study. With both doses the uptake was linear over the incubation times used, indicating that incubation times within these ranges could be used to calculate the initial velocity of the uptake. For the subsequent kinetic study 7 min incubation time was used to calculate the initial rate of uptake of ^{3}H-norepinephrine.

The Michaelis-Menten analysis of the initial rate of uptake of norepinephrine with various concentrations of the catecholamine is shown in Fig. 2. Linear regression analysis of the results from ACTH pretreated and control atria gave straight lines with the following linear correlation coefficients, r=1.000 (ACTH) and r=.999 (control), indicating that the uptake process was obeying Michaelis-Menten kinetics. Linear regression lines were fitted by the method of least squares. A test for parallelism on the two regression equations indicated that the two lines were not parallel, the two gradients (V_{max} for ACTH pretreated and control) being significantly different ($p<0.001$). The maximum rate of uptake for the ACTH pretreated atria was 1239 DPM/mg/min and for the control atria 1689 DPM/mg/min. The values of K_m calculated using the regression equations (±95% confidence limits) were 22.2 (± 2.1)$\times 10^{-8}$ M for the ACTH atria and 24.1 (± 2.3)$\times 10^{-8}$ M for the control atria.

The dose-response curve for the effect of (1–24)ACTH on the uptake of ^{3}H-norepinephrine is shown in Fig. 3. Both the 8.5 and 85×10^{-9} M dose of ACTH brought about a significant

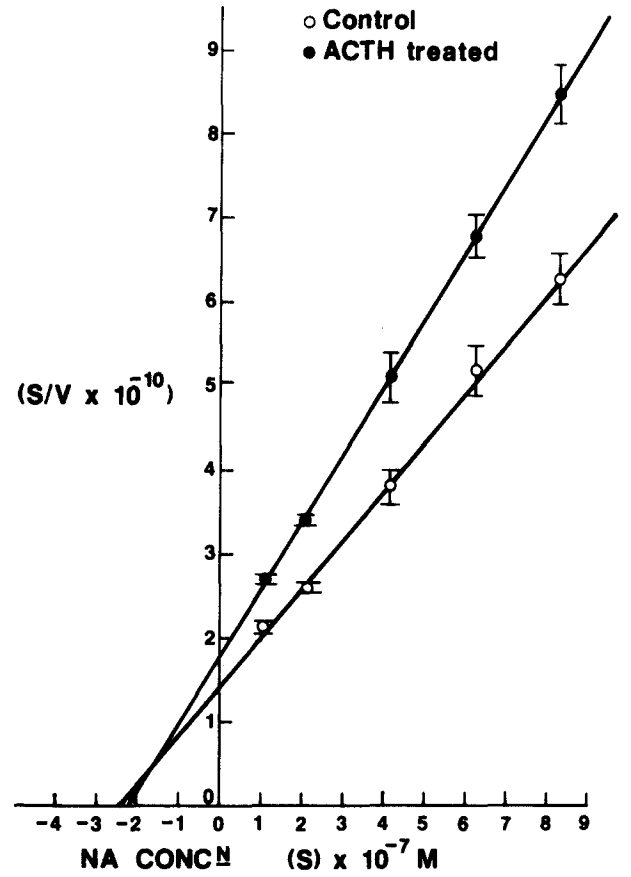

FIG. 2. Michaelis-Menten analysis of the initial rate of uptake of ^{3}H-norepinephrine in the presence and absence of 8.5×10^{-9} M ACTH. S=incubation concentration of norepinephrine (molar bath conc.). V=initial rate of norepinephrine uptake by spontaneously beating atria (DPM/mg/min) Intercept on baseline=$-K_m$, slope=$1/V_{max}$. V_{max}=maximum rate of uptake. Each point represents the mean value, vertical bars denote ±S.E.M.

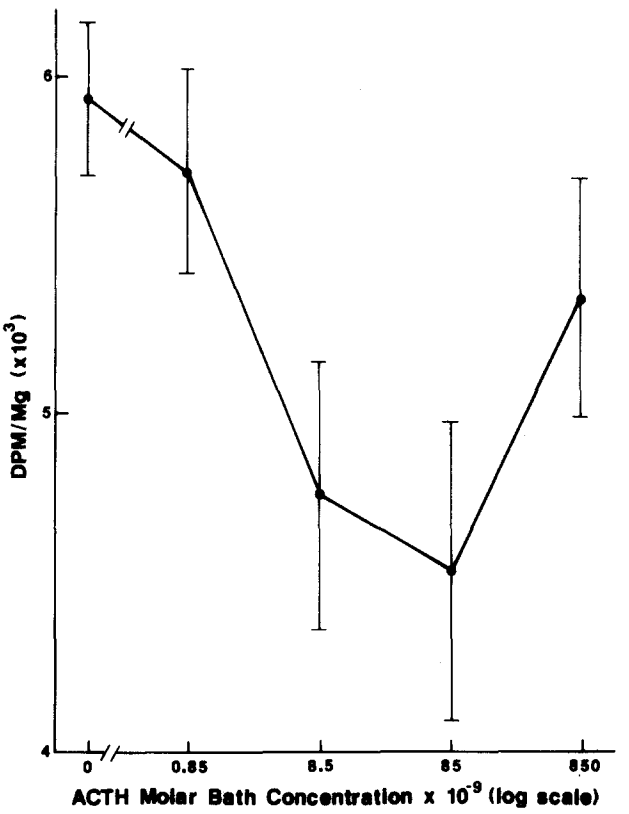

FIG. 3. The effect of various nominal bath concentrations of (1–24)ACTH on the uptake of ^{3}H-norepinephrine by isolated spontaneously beating rat atria. Each point represents the mean value of 5 atria. Vertical bars represent ±S.E.M. Both the 8.5 and 85×10^{-9} M concentrations gave significantly different values from the control (0) value (unpaired t-test). Ordinate: uptake of radioactivity (DPM/mg); abscissa=molar bath concentration of (1–24)ACTH.

reduction in the uptake of ^{3}H-norepinephrine into rat atria when compared to the control unpretreated atria (unpaired t-test; $p<0.05$ and $p<0.03$, respectively). At nominal concentrations of (1–24)ACTH below 8.5×10^{-9} M and above 85×10^{-9} M the reduction in ^{3}H-norepinephrine uptake was not significant ($p>0.05$). The form of this dose-response curve is similar to that reported for the potentiation of myocardial sensitivty to norepinephrine by (1–24)ACTH [5].

Measurements of the acutal bath concentration of (1–24)ACTH following the addition of ACTH at the nominal concentrations of 8.5 and 85×10^{-9} M are shown in Table 3. The actual concentration of (1–24)ACTH in the organ bath was considerably less than the nominal concentration of the hormone added. The percentage loss of (1–24)ACTH was greatest with the higher concentration added (85×10^{-9} M). While the greatest percentage loss occurred within the first minute, the loss continued throughout the time period studied (10 min).

TABLE 3

ACTUAL MOLAR BATH CONCENTRATION ($\times10^{-10}$ M) OF (1–24)ACTH AT VARIOUS NOMINAL BATH CONCENTRATIONS OF THE HORMONE (MEAN ± S.E.)

Time of Sampling	Nominal Dose of (1–24)ACTH Added to Bath (Molar Bath Concentration × 10^{-9} M)	
	8.5	85
1 min	5.11 ± 0.59 (5)	15.96 ± 1.72 (6)
5 min	2.88 ± 0.37 (5)	8.90 ± 0.82 (8)
10 min	1.67 ± 0.21 (8)	2.96 ± 0.48 (8)

Numbers in parentheses = sample size.

DISCUSSION

The present study has shown that (1–24) ACTH will inhibit the uptake and retention of ^{3}H-norepinephrine into the rat atrium. The inhibition appears to reflect an interference in the rate of uptake into storage sites rather than a change in the storage itself. While the accumulation of radioactivity was initially less in atria pretreated with (1–24)ACTH, if incubation was prolonged the levels of activity in both control and ACTH pretreated tissues were not significantly different. This finding is confirmed by the kinetic studies. Atria

pretreated with (1–24)ACTH showed a significant lowering of the maximum rate of uptake (V_{max}) but no change in the affinity of norepinephrine for the uptake mechanism (K_m). It would appear that the norepinephrine is competing for the same uptake sites, but that in the presence of (1–24)ACTH there are fewer sites available.

The concentrations of norepinephrine used in this study were well within the range of concentration found by Iversen [15–17] and Farnedo and Malmfors [11] to be accumulated almost exclusively by the Uptake 1 mechanism. This would suggest that (1–24)ACTH is affecting mainly neuronal uptake. Such an hypothesis is confirmed by the kinetic study. The K_m for the control and (1–24)ACTH pretreated tissues were 24.1×10^{-8} M and 22.2×10^{-8} M, respectively. Iversen [15] found that for Uptake 1 (neuronal uptake) the K_m for l-norepinephrine was 26.6×10^{-8} M.

Since the most important mechanism for the inactivation of the catecholamines is their active reuptake back into neuronal stores [1,20], inhibition of this uptake process would greatly increase the availability of the amine to its post-synaptic receptor site. Such an increased availability would explain the ACTH-induced enhanced myocardial sensitivity to the catecholamines [5]. Also, as ACTH was more potent than the glucocorticoids in inducing such an enhanced myocardial sensitivity, ACTH, at least initially, may be the main hormone involved in the stress induced change in myocardial sensitivity [3] and not the glucocorticoids as previously suggested [3,4].

While the nominal bath concentrations of (1–24)ACTH used in this study are comparable with those used by Bassett *et al.* [5] to show an ACTH-induced increase in myocardial sensitivity to norepinephrine, they are well above the physiological levels reported to occur following exposure to stress [7, 12, 22]. There is, however, a considerable loss of ACTH (Table 3) due to adsorption on the glass walls of the organ bath and breakdown of the labile hormone [8]. The actual bath concentration of (1–24)ACTH at a nominal concentration of 8.5×10^{-9} M, was found to be close to the range reported for plasma ACTH following exposure to a stressor; 500 pg ml^{-1} [7] to approximately 1300 pg ml^{-1} [12]. In the present experiment, with a nominal bath concentration of 8.5×10^{-9} M (1–24)ACTH, the actual bath concentrations were 5.1×10^{-10} M (1500 pg ml^{-1}) at 1 min, 2.9×10^{-10} M (850 pg ml^{-1}) at 5 min, and 1.7×10^{-10} M (500 pg ml^{-1}) at 10 min.

ACTH has not been reported previously to have any effect on the neuronal uptake of catecholamines, nor to have receptors in cardiac tissue. However, ACTH binding sites in the rat median eminence have been localized in vivo [25]. These binding sites were localized to axon terminals in the region, and it is possible they may be the receptors associated with the neuronal uptake process. Further indirect evidence in support of ACTH's action in inhibiting neuronal uptake are the reports that ACTH can enhance stimulation-evoked release of norepinephrine in sympathetic neurons [13], and that the decreases in β-receptor function noted after ACTH treatment [19] may be secondary to a hormone-induced change in synaptic concentration of neurotransmitter [9]. Duman, Andree, Kendall and Enna [9] suggested that ACTH administration modifies the norepinephrine-stimulated cyclic nucleotide system indirectly, perhaps through an action on the pre-synaptic neuron. ACTH has been shown to produce a selective reduction in the function of adrenergic receptors within the rat brain, an action which was similar to that seen with the norepinephrine reuptake inhibitors desmethylimipramine and imipramine [10,24]. ACTH administration causes a significant reduction in norepinephrine-stimulated cAMP accumulation (a biochemical measure of β-adrenergic receptor activity), whether given alone or in combination with imipramine [10]. ACTH will also facilitate the imipramine-induced reduction in β-receptor binding [10]. It is believed that desmethylimipramine and imipramine modify noradrenergic activity by inhibiting transmitter reuptake, and that the decline in β-adrenergic receptor number following chronic treatment with these agents is the result of excess receptor occupancy brought on by the increase in synaptic concentration of the catecholamine [10]. While ACTH does not affect antagonist (^{3}H-dihydroalprenolol; ^{3}H-DHA) attachment to the β-adrenergic receptor, it has been shown to shift the potency of the agonist (norepinephrine) to displace the specifically bound ^{3}H-DHA [10]. ACTH is also known to be capable of enhancing noradrenergic neuronal activity [21]. Such findings are consistent with the hypothesis that the receptor effects reported for ACTH are secondary to its ability to increase the availability of norepinephrine due to an inhibition of the reuptake process.

An inhibition of neuronal uptake of catecholamines by ACTH may explain the observations that ACTH administration depletes the catecholamine content of certain central nervous system nuclei, particularly those of the hypothalamus [14], and that the conversion of tyrosine to dopamine is increased by (1–24)ACTH [18]. Depletion of aminergic stores by inhibiting reuptake would result in the intra-cellular constraints on tyrosine hydroxylase (the rate limiting enzyme in the synthesis of norepinephrine) being lifted, leading to an enhanced synthesis. ACTH secretion is associated with increased hypothalamic norepinephrine turnover rates in rats [23].

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