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Adrenocorticotropin Binding Sites in Rat Cardiac Tissue

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MEHRABANI, P. A. AND J. R. BASSETT. *Adrenocorticotropin binding sites in rat cardiac tissue.* PHARMACOL BIOCHEM BEHAV **35**(1) 99–103, 1990.—Using the ACTH analog [^{125}I-Tyr^{23},Phe^{2},Nle^{4}] ACTH(1–24), the existence of specific binding sites for ACTH in atrial membrane preparations was demonstrated. The dissociation constants (K_d), determined by Scatchard analysis, were not significantly different for membrane preparations of adrenal gland or atrial tissue (being 6.40×10^{-12} M and 8.86×10^{-12} M respectively). No binding was observed to membrane preparations from kidney or lung. While the binding of the ACTH(1–24) analog to atrial membranes was inhibited by ACTH(1–24), it was not affected by norepinephrine or epinephrine. It was proposed that the ACTH(1–24) analog may bind to sites located on the adrenergic nerve endings associated with the cardiac tissue, and that such binding would interfere with the neuronal reuptake of the catecholamines.

Adrenocorticotropin Binding sites Atria Neuronal uptake ACTH analog

BASSETT, Strand and Cairncross (2) found that adrenocorticotropic hormone (ACTH) induced an enhanced myocardial sensitivity to both norepinephrine and epinephrine. While previous studies had suggested that ACTH's action in this regard may be attributed to its ability to inhibit the uptake and retention of the catecholamines by the adrenergic nerve endings (1,2), it is only recently that such an inhibition by ACTH(1–24) has been confirmed in atrial tissue (11). Since ACTH(1–24) does inhibit neuronal uptake in rat cardiac tissue, it is reasonable to suggest that receptors for the peptide hormone may exist in this tissue. ACTH binding sites in the rat median eminence have been localised in vivo (16). These binding sites were localised to axon terminals in the region, and it is possible that they may be the receptors associated with the neuronal uptake process. Outside the CNS it is becoming apparent that cells other than those in the adrenal gland can bind ACTH, including adipocytes (12) and lymphocytes (9). However, so far no specific binding sites for ACTH in rat cardiac tissue have been reported.

The present study was undertaken to investigate the existence of specific binding sites for ACTH(1–24) in atrial tissue of the rat using crude membrane preparations. Despite numerous attempts to study the binding of ACTH to its receptors by direct binding methods, detection and characterisation of the receptors has proved difficult (5).

The major difficulties encountered are the extraordinary propensity of the hormone to bind to inert materials and nonreceptor components of the target tissue, and the low biological potencies of the radiolabelled ACTH preparations (5). Loss of biological potency of ACTH results from the introduction of an iodine atom into tyrosine residue in position 2 and the oxidation of the methionine in position 4. The synthesis of an ACTH analog in which the tyrosine^{2} in replaced by phenylalanine and the methionine^{4} by norleucine produces a peptide equipotent with ACTH in stimulating steroidogenesis in adrenocortical cells, and where iodination retained full biological potency (5,8). The ACTH analog was used in this study.

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.

Radiolabelled ACTH analog, [^{125}I-Tyr^{23},Phe^{2},Nle^{4}] ACTH(1–24)

The ACTH analog [Phe^{2},Nle^{4}]ACTH(1–24) (Australian Laboratory Services, Sydney) was radioiodinated by the modified chloramine T method of Djura and Hoskinson (6), and purified using Sep-Pak reverse-phase cartridges (Waters Associates, Sydney); purity being confirmed using high performance liquid chromatography (7). The specific radioactivity of the batches of the [^{125}I]ACTH analog varied between 460 to 1420 Ci/mmol.

Atria and Adrenal Gland Membrane Preparations

Crude membrane preparations were prepared by a modified

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method of Durand (7). Animals were killed by cervical dislocation and exsanguinated. Both adrenal glands and the atria were rapidly dissected out, weighed and stored in liquid nitrogen. Five pairs of adrenals or 5 sets of atria were homogenised in 10 ml of 10 mM Tris HCl buffer containing 0.25 M sucrose at 4°C. The homogenate was first centrifuged at 4°C for 10 min at 600 × g to remove unbroken cells and nuclei. The supernatant was then centrifuged at 20,000 × g for 30 min at 4°C. The pellet was resuspended in 6 ml of cold 20 mM Tris buffer containing no sucrose. The suspension was used as the crude membrane preparation in subsequent studies. The protein content of the suspension was assayed using the Bio-Rad microassay procedure (Bio-Rad, Sydney, Australia). This assay is based on the method of Bradford (4).

[^{125}I]ACTH(1–24) Analog Binding Assay

The assay was performed in polystyrene tubes 10 × 55 mm. Membrane preparations (200 μl) in 20 mM Tris buffer containing 1% bovine serum albumin (BSA) (protein concentration; atria 0.7 mg/ml; adrenal gland 0.39 mg/ml) were incubated at 4°C with 50 μl of [^{125}I]ACTH(1–24) analog (80,000 cpm) and 200 μl of 20 mM Tris buffer containing 1% BSA for 2, 5 and 10 min. Incubations were routinely conducted in triplicate. After the selected incubation period, 0.5 ml of cold Tris buffer containing 0.1% polyethylene glycol was added to the incubation mixture, vortexed, then centrifuged at 10,000 × g for 10 min at 4°C. An aliquot of the supernatant (500 μl) was taken and counted in a gamma counter. By subtracting the total activity in the supernatant from the activity added, the activity of the [^{125}I]ACTH(1–24) analog bound to the membrane preparation was obtained. This represented the total activity bound (both specific and nonspecific binding). Nonspecific binding was assessed by replacing the 200 μl of Tris buffer (1% BSA) in the incubation mixture with an equal volume of buffer containing an excess (100 μM) unlabelled ACTH(1–24) (Synacthan, Ciba-Geigy Australia Ltd.).

Tissue Specificity in the Binding of [^{125}I]ACTH(1–24) Analog to Membrane Preparations

To determine whether the binding phenomenon was specific for atrial and adrenal membranes, the binding of [^{125}I]ACTH(1–24) analog to membrane preparations from several different tissues was compared. All membrane preparations were prepared identically. Incubations were carried out for 2 min, as described above, with membranes from rat atria, adrenal glands, kidney and lung (protein concentrations ranging from 400 to 700 μg/ml). The activity of [^{125}I]ACTH(1–24) analog specifically bound was determined and expressed as counts/min/mg of protein.

Displacement of [^{125}I]ACTH(1–24) Analog Binding

Since it had previously been reported that ACTH(1–24) would inhibit the neuronal uptake of the catecholamines (11), the ability of norepinephrine and epinephrine to inhibit the binding of the [^{125}I]ACTH(1–24) analog to atrial membrane preparation was investigated. In this series of experiments the amount of isotope bound (total bound) was measured in the presence of increasing concentrations of either unlabelled ACTH(1–24), norepinephrine, or epinephrine. The final concentration of the inhibitor in the incubation mixture ranged from 4.26×10^{-4} M to 4.26×10^{-7} M. The incubation time was 2 min. The results are expressed as a percentage of total bound in the absence of the inhibitor.

Scatchard Analysis of [^{125}I]ACTH(1–24) Analog Binding

To characterise the properties of both the adrenal and atrial binding sites, the binding of the [^{125}I]ACTH(1–24) analog to membrane preparations was measured using variable amounts of the radiolabelled analog. The method was essentially the same as that described previously with the exception that membrane preparations (200 μl) were incubated with varying volumes of [^{125}I]ACTH(1–24) analog (7, 10, 15, 20, 30, 40, 50 and 60 μl) together with 20 mM Tris buffer to give a final volume of 750 μl. The ratio of bound to free [^{125}I]ACTH(1–24) analog was calculated and plotted against the amount of labelled analog bound. Scatchard analysis (14) was carried out to determine the apparent dissociation constant, K_d.

The relationship between the bound/free ratio and the total [^{125}I]ACTH analog bound was found not to be linear over the range of ACTH concentrations investigated (see Figs. 4 and 5) indicating at least two populations of binding sites in both adrenal and atrial tissue. Subsequent studies concentrated on the high-affinity binding sites by using only the lower concentrations of the ACTH analog (5, 7, 9, 11, 13, 15, 17 and 20 μl; the final incubation volume remaining at 750 μl). Under these conditions binding to only a single population of binding sites was observed on Scatchard analysis.

RESULTS

The time course for the binding of [^{125}I]ACTH(1–24) analog to both adrenal and atrial membrane preparations is shown in Fig. 1. Maximum binding at 4°C with both membrane preparations occurred within 2 min, the earliest time at which measurements were made. This is in agreement with the observations of Ways, Zimmerman and Ontjes (17) who found also that maximum binding of [^{125}I]ACTH(1–24) to adrenal membrane preparation at 4°C occurred within 2 min. With atrial membrane there was no significant change in binding over the time period studied (unpaired t-test, $p > 0.05$). However, with adrenal membranes, total binding declined slowly, so that by 10 min incubation the total binding was significantly less than at either 2 or 5 min (unpaired t-tests, $p < 0.05$ in both cases). While, with the present data, it is not possible to say why binding to the adrenal membrane declined with time, a similar observation has been reported by Saez *et al.* (13). Saez suggested that the decline could be due to either degradation of the ligand or a denaturation of the receptor. A similar pattern was seen if the percentage of [^{125}I]ACTH(1–24) analog specifically bound to the membrane preparations was plotted against time.

Through this series of experiments the percentage of the [^{125}I]ACTH(1–24) analog bound to membrane preparations was 68.8 ± 0.1% (mean ± S.E.M.), of which 48.6 ± 0.4% was specifically bound. The ratio of specifically bound to total bound did not vary significantly throughout these experiments, and there was no significant difference between atrial and adrenal preparations in terms of the percentage total bound or the percentage specifically bound.

The binding of [^{125}I]ACTH(1–24) analog to membrane preparations from a number of tissues is shown in Fig. 2. The results are expressed as the activity of the labelled analog specifically bound to the membrane preparation per mg of protein in the incubation tube. Although the adrenal and nonadrenal tissues all showed some nonspecific binding of the radiolabelled analog, only the adrenal and atrial preparations showed any appreciable binding which was displaceable with unlabelled ACTH(1–24). Both kidney and lung tissue showed little specific binding of [^{125}I]ACTH(1–24) analog. The [^{125}I] analog specifically bound/mg protein was significantly greater in the adrenal tissue when compared to atrial tissue (unpaired t-test, $p < 0.001$).

The displacement of the [^{125}I]ACTH(1–24) analog, bound to

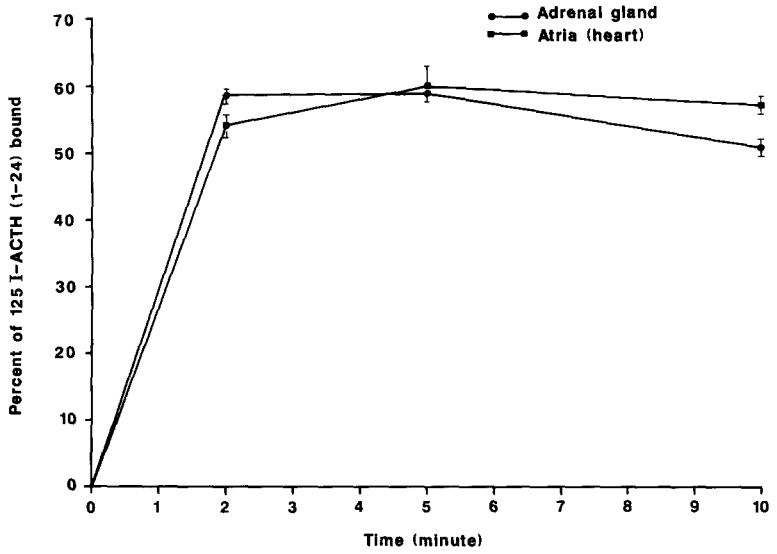

FIG. 1. Time course of binding of [^{125}I]ACTH(1–24) analog to atrial and adrenal membrane preparations. Each point represents the mean of five determinations. The horizontal bars denote ±S.E.M.

atrial membranes, by catecholamines and ACTH(1–24) is shown in Fig. 3. ACTH(1–24) significantly inhibited the binding of the labelled analog [one-way analysis of variance, $F(3,10)=21.45$, $p<0.01$]. At concentrations of ACTH(1–24) of 4.26×10^{-5} M and 1.70×10^{-5} M, the binding of the labelled analog was significantly reduced (unpaired t-test, $p<0.01$ in both cases). In this study, the greatest inhibition was observed at the concentration of 4.26×10^{-5} M, where the binding of [^{125}I]ACTH(1–24) analog was reduced to 46.7% of the total isotope bound in the absence of inhibitor. This observation is in close agreement with the finding reported earlier that an excess of unlabelled ACTH(1–24) (100 μM) will inhibit the specific binding of the labelled analog and that the percentage of the total bound isotope specifi-

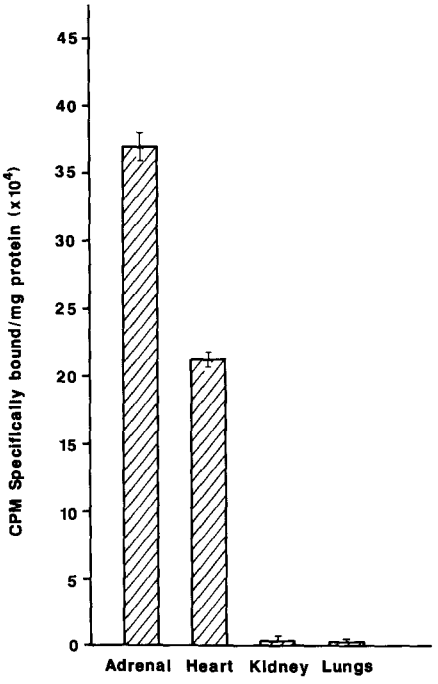

FIG. 2. Specific binding of [^{125}I]ACTH(1–24) analog to membranes obtained from a number of rat tissues. The membrane preparations from kidney and lung were prepared as described for atrial and adrenals. The height of the columns represents the mean of 3 preparations, the horizontal bars denote ±S.E.M.

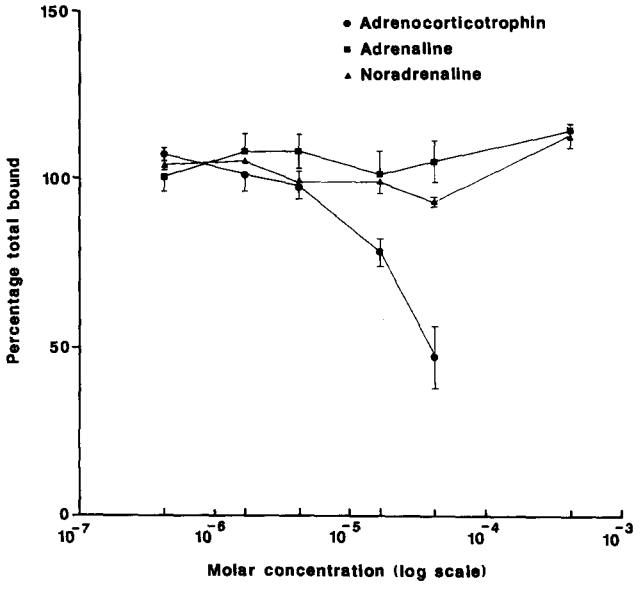

FIG. 3. The inhibitory effects of various concentrations of ACTH(1–24), norepinephrine and epinephrine on the binding of [^{125}I]ACTH(1–24) analog to atrial membrane preparations. The ordinate represents the effect as a percentage of the total bound analog in the absence of inhibitory agents. Each point represents the mean of 3 preparations; horizontal bars denote ±S.E.M. ●: ACTH(1–24); ■: epinephrine; ▲: norepinephrine.

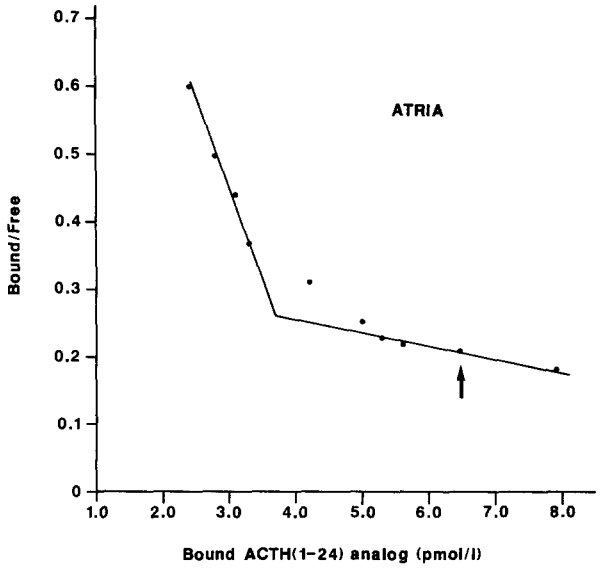

FIG. 4. Scatchard plot for the binding of [^{125}I]ACTH(1–24) analog to an atrial membrane preparation at 4°C. Linear regression analysis on the 4 points representing the lowest quantities of bound ACTH analog gave a regression coefficient of r = .986, and an apparent dissociation constant K_d (the negative reciprocal of the slope) of 5.98×10^{-12} M. The arrow indicates the amount of labelled analog bound in previous experiments.

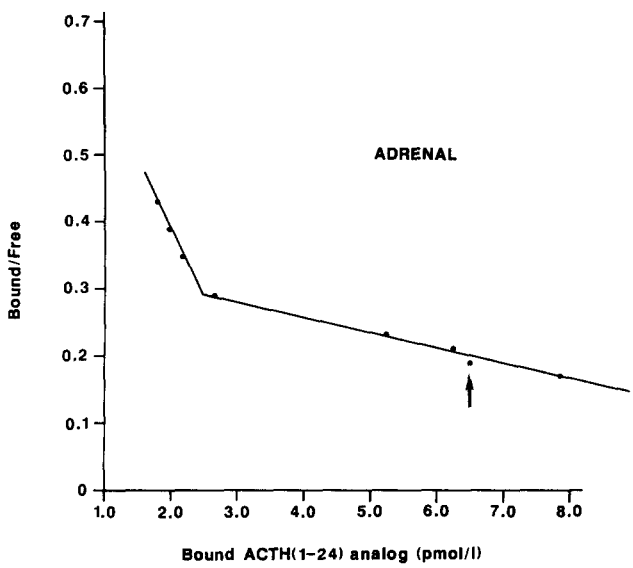

FIG. 5. Scatchard plot of the binding of [^{125}I]ACTH(1–24) analog to an adrenal membrane preparation at 4°C. Linear regression analysis of the 3 points representing the lowest quantities of bound ACTH analog gave a regression coefficient of r = .995, and an apparent dissociation constant K_d of 5.97×10^{-12} M. The arrow indicates the amount of labelled analog used in the previous experiments.

cally bound was 48.6%. The catecholamines, norepinephrine and epinephrine did not significantly alter the binding of the [^{125}I]ACTH(1–24) analog to atrial membrane preparations over the dose range studied, 4.26×10^{-7} M to 4.26×10^{-4} [one-way analysis of variance; norepinephrine, $F(5,12) = 2.85$, $p > 0.05$; epinephrine, $F(5,12) = 1.06$, $p > 0.05$].

A Scatchard plot of the binding of [^{125}I]ACTH(1–24) analog to atrial tissue is shown in Fig. 4, and to adrenal tissue in Fig. 5. In both cases the relationship between the bound/free ratio and total ACTH analog bound is not linear over the range of ACTH analog concentrations examined, indicating at least two populations of binding sites. In order to investigate the high-affinity binding sites in both tissues, we subsequently concentrated on the binding at lower concentrations of the [^{125}I]ACTH(1–24) analog. These concentrations corresponded to the initial steep portions of the Scatchard plots shown in Fig. 4 and 5. On the basis of these studies the mean (±S.E.M.) K_d for the atrial membrane (n = 5) was found to be $8.25 \pm 0.98 \times 10^{-12}$ M, and for the adrenal membranes (n = 5) $6.57 \pm 0.51 \times 10^{-12}$ M. An unpaired *t*-test showed no significant difference between these two values ($p > 0.05$).

DISCUSSION

While previous studies have indicated the possible importance of ACTH as a modulator of catecholamine activity in cardiac tissue (1, 11, 15), there have been few reports suggesting the existence of specific ACTH receptors in such tissue. The evidence for ACTH receptors in the heart has generally been indirect, involving studies utilizing cholinergic and adrenergic stimulation and blockade (15). Zeiler, Strand and El-Sherif (19) were able to show that [^{125}I]ACTH(1–24), when incubated with canine left atrial sections, would bind to sites in cardiac tissue, a finding which is in agreement with those of the present study using rat atrial membrane preparations. Binding of the ACTH(1–24) analog was only observed to any appreciable extent in membrane preparations obtained from adrenal and atrial tissue. Little binding was observed to membrane preparations obtained from kidney or lung tissue. Ways, Zimmerman and Ontjes (17) also demonstrated the absence of ACTH(1–24) binding sites in kidney, lung and liver preparations.

The present study does not determine on which cell type in the atrium the ACTH receptors are located. It is known that ACTH alone does not affect the mechanical characteristics of the heart (15). The positive inotropism induced by ACTH is dependent upon the release of endogenous norepinephrine (15), or the administration of exogenous norepinephrine (2,19).

Investigating the possible mechanisms by which ACTH may exert its affect on atrial tissue, Zeiler *et al.* (19) proposed that ACTH may affect atrial contractility by a direct action on the cardiac muscle itself. They suggested that while ACTH does not appear to affect cardiac cyclic AMP directly, it may affect other components of the chain of intracellular reactions triggered by cyclic AMP. On the other hand, Mehrabani and Bassett (11) reported that ACTH(1–24) inhibited the neuronal reuptake of norepinephrine in cardiac tissue, and proposed that the ACTH-induced potentiation of the inotropic actions of the catecholamines (2) was due to such an inhibition of reuptake. If this were the case then the ACTH binding sites in cardiac tissue may be located on the adrenergic nerve terminals associated with the cardiac tissue, and not on the heart muscle itself. In the median eminence ACTH binding sites have been localised to axon terminals in the region (16), and such receptors may be associated with the adrenergic uptake process.

It is of interest to note that while ACTH(1–24) inhibits the neuronal uptake of the catecholamines (11), the catecholamines do not appear to interfere with the binding of ACTH in cardiac tissue. Recent studies have shown the ACTH receptor to be a complex structure. In the mouse adrenal tumor it is composed of 4 separate protein subunits, the ability to specifically bind [^{125}I]ACTH being localised to only one of these subunits (3).

Studies of the structure-activity relationship of ACTH in

adrenal tissue have shown that the peptide sequences necessary for the binding and biological activity of the hormone are localised in different parts of the molecule. Although there is an overlap of function, the N-terminal sequence appears to be essential for its biological action while the 11–24 sequence at the C-terminal is important for binding (13). Ways *et al.* (17) also concluded that for ACTH's action on the adrenal gland the structural requirements for biological activity and for receptor binding are not identical. If the ACTH binding sites in cardiac tissue are similar to those in adrenal tissue, then while the binding of ACTH to its receptor may inhibit the neuronal reuptake of the catecholamines (the biological response), the catecholamines need not affect the binding of ACTH to its receptor. In the present study, Scatchard analysis of the binding of the ACTH(1–24) analog to both adrenal and atrial membranes gave similar K_d values, indicating that indeed the binding sites in both tissue may be similar.

The dissociation constant obtained for the high-affinity binding sites of the adrenal membrane preparation is in close agreement with that obtained by Gallo-Payet and Escher (8) using the same (^{125}I-Tyr^{23},Phe^{2},Nle^{4}]ACTH(1–24) analog and isolated rat adrenal cells ($K_d = 1.0 \times 10^{-11}$ M for fasciculata cells). However, using a similar ACTH analog, [^{125}I-Tyr^{23},Phe^{2},Nle^{3}]ACTH(1–38) and isolated rat adrenal cells, Buckely and Ramachandran (5) reported a K_d of 1.4×10^{-9} M. As suggested by Gallo-Payet and Escher (8), the marked differences in K_d values may be associated with differences in cell preparation. Buckely and Ramachandran (5) assayed their cells after 18 hr of primary culture at 37°C, whereas Gallo-Payet and Escher (8) tested freshly harvested cells, reporting visible decrease in binding capacity after several hours of primary culture. The membrane preparations used in the present study were all freshly prepared. The K_d values obtained with the ACTH analog also are in agreement with values obtained for rat adrenal cells using normal [^{125}I]ACTH(1–39) (10,18).

In summary, using the ACTH analog [^{125}I-Tyr^{23}, Phe^{2},Nle^{4}]ACTH(1–24), it has been possible to demonstrate the existence of specific binding sites for ACTH in atrial membrane preparations. While the binding of the ACTH analog was inhibited by ACTH(1–24), it was not affected by the catecholamines, norepinephrine or epinephrine. It is proposed that ACTH may bind to sites on the adrenergic nerve endings and not on the cardiac muscle itself. In so doing, ACTH would interfere with the neuronal reuptake of the catecholamines, and thus explain the ACTH-induced enhanced myocardial sensitivity to both norepinephrine and epinephrine.

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