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Regional High-Affinity [^{3}H]Choline Accumulation in Cat Forebrain: Selective Increase in the Caudate-Putamen After Corticosteroid Pretreatment

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SUMMARY

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The effect of acute and chronic gluco- and mineralocorticoid treatment on high-affinity [^{3}H]choline accumulation (HACA) was investigated in cat brain synaptosomes. HACA in forebrain synaptosomes was found to be Na^+- and energy-dependent, hemicholinium-3 sensitive ($K_i \cong 2.5$ nM), and of high affinity (apparent $K_T = 0.43 - 0.63$ μM). The rank order of regional HACA at 0.04 μM choline (4 min at 37°) was: caudate-putamen-anterior perforated space > hippocampus > prefrontal neocortex. HACA in caudate-putamen synaptosomes was significantly elevated 37–75% after daily treatment for one week with either triamcinolone diacetate (8 mg/kg), hydrocortisone acetate (4 or 32 mg/kg), or deoxycorticosterone acetate (32 mg/kg), but not with 11α-epicortisol (8 mg/kg), a biologically inert epimer of hydrocortisone. Treatment-induced increases in caudate-putamen HACA were attributable to an increase (45–53%) in the maximal transport velocity (V_{max}) and not in the apparent transport constant (K_T). Significant increases in HACA were not seen in the hippocampus-fornix, anterior perforated space, or prefrontal cortex. Acute treatment of cats with a single intravenous dose of methylprednisolone sodium succinate (90 mg/kg) produced 75–84% increases in HACA in caudate-putamen and hippocampus-fornix three hours after treatment. At 24 hours HACA was increased (70 & 76%) in the caudate-putamen and anterior perforated space. Increases in HACA found in the caudate-putamen after *in vivo* treatment could not be produced by *in vitro* addition of steroids or alterations of the Na^+/K^+ environment. We conclude that the velocity of high-affinity transport of choline into cat brain synaptosomes is strongly and selectively increased in the caudate-putamen after acute or chronic gluco- or mineralocorticoid treatment. The possible explanations for this selectivity are discussed with reference to the neurochemistry and neuropharmacology of the caudate-putamen.

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INTRODUCTION

High-affinity accumulation of [^{3}H]choline (HACA)[2, 3] in synaptosomes is saturable, Na^+-dependent, localized in central and peripheral cholinergic neurons, and appears restricted to the nerve terminal. Most tissues lacking cholinergic innervation contain no Na^+-dependent high-affinity component (1–3). HACA is coupled to choline acetyltransferase activity, forms acetylcholine (Ach) and can support its turnover (2–4). Recent experiments in rat hippocampus have shown that the accumulation of this Ach precursor is dependent on Ach release, intrasynaptic Ach levels (5) and is coupled to neuronal activity (2, 3). All of these findings have led investigators to suggest that HACA in nerve terminals is the rate-limiting regulatory step in the synthesis of Ach.

In this investigation, we examined HACA in brain synaptosomes[4] from cats pretreated with high daily doses of gluco- or mineralocorticoids. Previous work on the effect of adrenocorticoids on the nervous system prompted this approach. In the peripheral nervous system, intensive treatment of cats with glucocorticoids greatly augments, in the cholinergic nerve terminals of soleus muscle, the excitability increase that follows high-frequency stimulation (50–400 Hz) (6). Also, in the cat's central nervous system, as in that of other species, glucocorticoids markedly increase excitability and reduce the seizure threshold of specific brain regions (7). In each instance, the consequence of intensive treatment is to provoke an abnormal bursting discharge of susceptible neurons. For cholinergic neurons so altered, an increase in transmitter turnover might be anticipated. Also central neurotransmitter uptake systems may be the primary sites of action of adrenocorticoids in the production of affective disorders (8).

We found a selective increase in HACA in the caudate-putamen from cats pretreated with adrenocorticoids.

[2] We have chosen to use the most correct term, high-affinity [^{3}H]choline accumulation; that use was suggested by several previous authors (e.g., 1), in preference to high-affinity choline uptake.

[3] The abbreviations ued are: HACA, high-affinity [^{3}H]choline accumulation; Ach, acetylcholine; DOCA, deoxycorticosterone acetate; APS, anterior perforated space, CP, caudate-putamen; HF, hippocampus-fornix; PFC, pre-frontal neocortex; HC-3, hemicholinium-3; CV, coefficient of variation; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

[4] Yamamura and Snyder (1) documented that the properties of HACA demonstrable in P_2 fractions are representative of properties of HACA in purified synaptosomes obtained from P_2 pellets after sucrose density gradient centrifugation.

MATERIALS AND METHODS

Steroid treatment. Chronically-treated cats were injected in the shoulder muscle once a day for 7 days with a depot formulation of steroid or vehicle (see below) (1–1.5 ml/kg). Normal cats received no treatment. On the first post-treatment day, cats were sacrificed, the brain quickly removed (~2 min), and immersed in ice-cold saline (0.9%). For acute treatment, methylprednisolone sodium succinate (90 mg/kg, *Solu-Medrol*; Upjohn) was given intravenously 3 or 24 hours before sacrifice. The following commercial depot formulations were administered intramuscularly: hydrocortisone acetate (*Hydrocortone acetate*, Merck, Sharp, & Dohme) and triamcinolone diacetate (*Aristocort*, Lederle). Depot suspensions for intramuscular administration of deoxycorticosterone acetate (DOCA; Sigma) and 11α-epicortisol (Steraloids, Inc., Wilton, N.H.) were made by suspending powders in peanut oil (Planter's).

For incubation procedures the following water soluble compounds were used: triamcinolone diacetate and triamcinolone-21-phosphate (courtesy of Dr. E. Cantrall of Lederle and Dr. S. Luciana of Squibb), hydrocortisone sodium phosphate (*Hydrocortone phosphate*, MSD), hemicholinium-3 (Aldrich), pyridoxal-5'-phosphate (Sigma), NADPH (Sigma) and choline chloride (Sigma).

Brain Dissection and synaptosomal preparation. The caudate-putamen and anterior perforated space (including nucleus accumbens, islands of Calleja, and olfactory tubercle) were chosen for their dense and intrinsic cholinergic innervation (9, 10). The hippocampus was chosen for its known extrinsic innervation from the medial septal nucleus (11). The prefrontal neocortex (sigmoid and coronal gyri, and propreus) was

chosen for its small expression of HACA in the rat (3).

Tissues were dissected on an ice-chilled platform, weighed, and homogenized in glass homogenizers with a Teflon pestle (clearance 125 nm) in 20 volumes ice-cold 0.32 M sucrose. Homogenates were centrifuged at 1,100 × *g* for 10 min at 4°. The formed pellet was discarded and the supernatant centrifuged at 18,000 × *g* for 15 min at 4°. The supernatant was discarded and the P_2 pellet, enriched in synaptosomes, resuspended (vortexed-rehomogenized) in a volume of ice-cold 0.32 M sucrose equal to that originally added to the tissue. Hereafter this resuspended P_2 pellet will be referred to as synaptosomes.

[^{3}H]Choline uptake assay. This procedure is similar to that described by previous workers (12). Temperature-dependent (37° vs 1°) high-affinity accumulation was measured at a final volume of 1 ml in 10 × 75 mm disposable glass tubes. Routine assays were performed by addition of 100 μl of the synaptosomal suspension in sucrose (~200 μg protein to 850 μl of Krebs-Ringer phosphate medium containing NaCl, 122 mM; KCl, 4.9 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; Na_2HPO_4, 12.8 mM; NaH_2PO_4, 3.0 mM [pH 7.4]; and dextrose [2 mg/ml], and varying concentrations of unlabeled choline chloride [see below]). Fifty microliters of [^{3}H]choline (Amersham/Searle, 6.4–13 Ci/mmole) made up in Krebs-Ringer medium was quickly added to yield a final [^{3}H]choline concentration of 0.032–0.052 μM. Final electrolyte concentrations during incubation were: Na^+, 137 mEq; K^+, 4.4 mEq; Ca^{2+}, 1.2 mEq; Mg^{2+}, 1.1 mEq. All tubes were vortexed at 1°. Triplicate sample tubes were transferred to a shaking water bath and incubated at 37° for 4 min. Duplicate 1° controls were held in ice water. Incubation was terminated by transferring sample tubes to ice water and shaking vigorously. Pellets of synaptosome were obtained by centrifugation at 4,500 × *g* for 15 min at 4°. The supernatant was carefully aspirated and the pellet immediately washed with 2 ml ice-cold 0.9% NaCl. Tubes were inverted to remove excess water. Tissue solubilizer (NCS, Amersham) was added (1 ml) and pellets were digested at 37° for 15 min. The tube contents were poured into scintillation vials and tubes were washed with 3.5 ml scintillation cocktail (PCS or ACS, Amersham). Radioactivity was measured in a total of 10 ml of cocktail by liquid scintillation spectrometry in a Packard Tri-Carb. Counting efficiency (36–40%) was determined in each assay in triplicate by addition of a known volume of calibrated [^{3}H]toluene. Uptake was linear between 1–5 min and 100–325 μg protein. The sample-to-blank ratio (37° vs. 1°) was at least 4:1.

Triplicate protein determinations were made by the method of Lowry *et al.* (13) using bovine serum albumin as a standard. The addition of sucrose (32 mM) did not shift the standard curve more than 10%.

For some *in vitro* experiments, synaptosomes were preincubated at 37° for 30 min with water-soluble steroid derivatives or potential enzyme cofactors (pyridoxal-5′-phosphate or NADPH). However, in experiments with hemicholinium-3 or altered Krebs-Ringer medium, synaptosomes were not preincubated. In Na^+-replacement experiments, Tris-HCl buffer (15.8 mM, pH 7.4 at 37°) replaced sodium phosphate buffer. Sucrose (274 mM), LiCl (137 mM), or CsCl (137 mM) replaced NaCl.

Lipid extraction of membranes. Synaptosomes were incubated as previously described except that incubation was carried out for 1 hr near the K_T for HACA (0.5 μM choline). After being formed into pellets, synaptosomes were superficially washed with 2 ml saline, lysed by vortexing in 2 ml distilled water, and centrifuged (4,500 × *g*, 15 min). Pellets were extracted in 5 ml Folch solution (chloroform-methanol, 2:1 v/v). The Folch extract was washed three times with 2 ml of 0.9% saline. Additional methanol was added (2 ml) to the Folch extract, which was then dried by a stream of nitrogen. The residue was resuspended in 1 ml NCS solubilizer and counted in scintillation cocktail.

Data analysis. Kinetic parameters (K_T and V_{max}) for [^{3}H]choline accumulation between 0.04–2.5 μM choline were obtained by both Lineweaver-Burk (1/v vs. 1/s) and Eadie-Hofstee (v/s vs. v) methods; the values compared favorably. However, we

chose to present Eadie-Hofstee estimates here because the method is less prone to artifactual distortions (14).

The hemicholinium-3 (HC-3) IC_{50} was determined by regression analysis of log-dose/percent inhibition data at 0.04 μM choline. K_i was calculated by simultaneous regression of 1/percent inhibition vs. 1/HC-3 concentration at three choline concentrations (0.04, 0.29, 0.54 μM) for four concentrations of HC-3 (0.1–100 nM).

Inferential statistics. For independent two-sample comparisons, the Mann-Whitney U-test was used with a two-tailed rejection region of $\alpha = 0.05$. For multiple mean comparisons the conservative, parametric Student-Newman-Keuls Multiple Range Test was used. To rule out slippage in k-sample comparisons, the Kruskal-Wallis Test (H) was used with a one-tailed $\alpha = 0.05$. To demonstrate a dose-related trend over a limited dose range, Joncheere's Test of an Ordered Alternative (15) was used to generate Kendall's correlation coefficient (τ) at a significance level of $\alpha = 0.05$.

RESULTS

[^{3}H]Choline incorporation into membranes. [^{3}H]Choline can be incorporated into synaptosomal and synaptic vesicle membrane phospholipids (16) as well as be transported into the synaptosomal lumen. Therefore, we first determined that the amount of [^{3}H]choline incorporated into membranes was a negligible portion of total [^{3}H]choline accumulation. Caudate-putamen or hippocampal synaptosomes were preloaded with [^{3}H]choline by a standard incubation at 0.04 μM (4 min at 37°). The synaptosomes were then osmotically lysed and washed as described in METHODS. The number of counts/min bound to the disrupted membrane pellet was 2–3% of the total counts/min accumulated by intact synaptosomes.

In a second set of experiments, we estimated the rate of incorporation of [^{3}H]choline into membrane lipids. Incubation was carried out near the K_T for high-affinity accumulation (0.5 μM) for 1 hr at 37°. Synaptosomes were then washed and osmotically lysed. The total lipid fraction of the membranes was extracted by a Folch procedure as described in METHODS. Under these conditions, [^{3}H]choline was incorporated into membrane lipids at a rate of 5–10 fmoles/mg protein/4 min[5], a rate approximately 100-fold slower than total [^{3}H]choline accumulation at 0.04 μM. Therefore, at concentrations of choline at or below the K_T of high-affinity accumulation, and for short incubation periods, membrane incorporation of [^{3}H]choline accounts for a negligible amount of the total [^{3}H]choline accumulation.

High-affinity attributes of choline accumulation. In practice, HACA has been defined by the following (2, 3): a) energy dependence; b) Na^+ dependence; c) K_T below 4 μM; d) sensitivity to HC-3 inhibition; e) a distribution coextensive with cholinergic nerve terminals. HACA in cat forebrain synaptosomes demonstrates all of these properties.

HACA was found to be energy dependent. Omission of dextrose from the incubation mixture decreased accumulation from 10–66% (mean 30%; $n = 4$). More importantly, HACA was strongly dependent on Na^+. Isosomotic replacement of NaCl by sucrose (274 mM), LiCl (137 mM), or CsCl (137 mM) in a Tris-buffered Krebs-Ringer incubation mixture completely inhibited [^{3}H]choline accumulation. Similar results have been reported in rat brain synaptosomes (2, 3). Interestingly, further increases in Na^+ concentration beyond normal (137 mM) produced little or no effect on HACA when compared to isosmotic controls (see Table 6 below). Lack of increased [^{3}H]choline accumulation in hypernatremic Krebs-Ringer medium has been reported in rat hippocampal synaptosomes (3). The remaining attributes of HACA are detailed below.

Forebrain distribution of synaptosomal high-affinity choline accumulation. The rate of HACA at 0.04 μM (4 min at 37°) into synaptosomes from four regions of the cat telencephalon is presented in Table 1. Synaptosomes of the anterior perforated space (APS, including the nucleus accumbens, islands of Calleja, and olfactory tubercle),

[5] [^{3}H]choline accumulation rates are expressed as pmoles of choline accumulated/mg protein/4 min to facilitate comparison with previously published data.

TABLE 1

Regional rates of high-affinity [^{3}H]choline accumulation in cat forebrain synaptosomes

Tissue	n^a	Accumulation rate[b]	X/PFC[c]
Anterior perforated space[d]	19	7.53 ± 0.65 (38) NS	4.1
Caudate-putamen	33	7.53 ± 0.35 (27) NS	4.1
Hippocampus-fornix	27	4.22 ± 0.33 (40)*	2.3
Prefrontal cortex	23	1.83 ± 0.14 (36)*	1.0

[a] Sample groups include untreated cats and vehicle-injected controls. These subgroups were combined after demonstration that they were drawn from the same population (see Table 3; $0.3 < p < 0.5$, Kruskal-Wallis one-way analysis of variance, $H = 1.50$, df = 2).

[b] [^{3}H]choline accumulation at 0.04 μM at 37° (pmoles/mg protein/4 min ± SEM). Number in parentheses is coefficient of variation (%).

[c] Ratio of given tissue (X) to prefrontal neocortex (PFC).

[d] Includes olfactory tubercle, islands of Calleja, and nucleus accumbens.

NS (nonsignificant) = $p > 0.05$.

* All tissue rates of accumulation are significantly different from all others ($p < 0.01$; Student-Newman-Keuls Test).

and the caudate-putamen (CP) exhibited equally-high rates of HACA. The hippocampus-fornix (HF), an area extensively investigated in the rat, shows only 56% of the activity of these other basal forebrain regions. The prefrontal neocortex (PFC) displays only 24% of the APS-CP activity. The mean accumulation rate of the CP and ASP are identical; all other paired comparisons are significantly different ($p < 0.01$, Student-Newman-Keuls Test).

Regional transport kinetics of high-affinity choline accumulation in forebrain. The maximal transport velocity (V_{max}) and apparent transport constant (K_T)[6] of HACA in forebrain synaptosomes were estimated by Eadie-Hofstee analysis (Table 4 below). A typical Eadie-Hofstee plot derived from CP synaptosomes of untreated and treated cats is shown in Figure 1. Eadie-Hofstee estimates of transport kinetic parameters yield significantly different V_{max}s and K_Ts in the CP, HF, and PFC ($p < 0.01$, Student-Newman-Keuls Test). This rank order is identical to the rank order of accumulation rates at 0.04 μM choline (Table 1). The K_T of the HF and PFC are identical; the K_T of the CP is higher ($p < 0.01$). The V_{max} of the HF is 47% and the K_T 70% of the CP values. The V_{max} of the PFC is 19% and the K_T 68% of the CP.

Hemicholinium-3 inhibition of HACA in forebrain synaptosomes. Inhibition by HC-3 is an important determinant of HACA.

[6] In all instances, K_T indicates an apparent K_T.

The concentration of HC-3 required to inhibit HACA 50% (IC_{50}) was determined by plotting percent inhibition of HACA over a concentration range of 0.1-nM to 100 nM HC-3. The mean IC_{50} at 0.04 μM choline for HC-3 in the CP and HF of untreated cats is shown in Table 2. The IC_{50} for both tissues is similar (2.5 nM). The K_i for HC-3 in the CP was determined between 0.1 and 100 M HC-3 at three choline concentrations below or at the K_T of high-affinity accumulation (0.04, 0.29, and 0.54 μM). Under these conditions, the K_i was 2.5 nM.

Effect of adrenocorticoids on high-affinity [^{3}H]choline accumulation in vitro. Hydrocortisone was studied because it is the primary, endogenous glucocorticoid secreted in the cat (17). Triamcinolone was included as a synthetic glucocorticoid because it is approximately four times as potent as hydrocortisone, has negligible mineralocorticoid action and less serum protein binding. Deoxycorticosterone acetate was used as a prototypic mineralocorticoid with little glucocorticoid action.

Triamcinolone-21-phosphate, hydrocortisone acetate, and 11-deoxycorticosterone acetate were assayed for their effect on CP HACA when added to the incubation mixture at a concentration of 10 μM. Steroid salts were solubilized in 0.1% ethanol-Krebs-Ringer or water-soluble salts were used directly. Incubation was carried out either under standard conditions (4 min at 37°), or with a preincubation of 30 min at

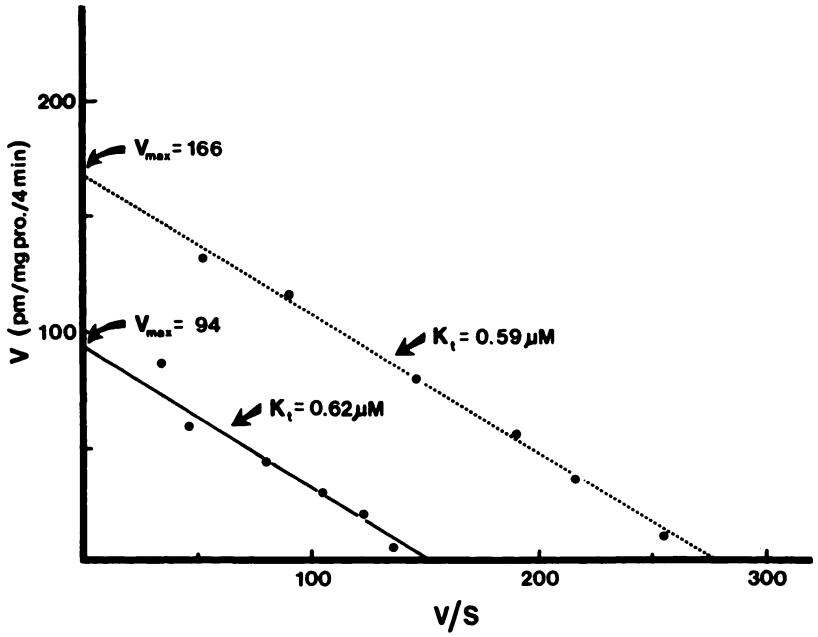

FIG. 1. *Representative Eadie-Hofstee plots of transport kinetics of [^{3}H]choline into cat caudate-putamen synaptosomes*

A solid line is fitted (by eye) to data from a single experiment using caudate-putamen synaptosomes pooled from two untreated cats. A broken line is fitted to data from a single experiment using caudate-putamen synaptosomes pooled from three cats treated daily for one week with hydrocortisone acetate (4 mg/kg *Hydrocortone acetate*, MSD).

TABLE 2

IC_{50} of hemicholinium-3 on in vitro *high-affinity [^{3}H]choline accumulation in cat forebrain synaptosomes*

Treated animals received hydrocortisone acetone (32 mg/kg) daily for one week. IC_{50} is expressed as mean concentration of hemicholinium-3 ± SEM required to inhibit 50% of [^{3}H]choline accumulation at 0.04 μM choline. Hemicholinium-3 concentration ranged between 0.1–100 nM. Numbers in parentheses are coefficients of variation (%).

Tissue	*n*	Untreated	*n*	Hydrocortisone
				32 mg/kg
Caudate-putamen	3	$2.9 \pm 0.2 \times 10^{-9}$ M (12)	2	$3.2 \pm 0.2 \times 10^{-9}$ M (7)
Hippocampus-fornix	2	$2.6 \pm 0.2 \times 10^{-9}$ M (11)	2	$2.6 \pm 0.1 \times 10^{-9}$ M (3)

37°. With exposure to any one of the steroids, CP synaptosomal accumulation of choline was not altered more than 5% in comparison to the same synaptosomes incubated with 0.1% ethanol-Krebs-Ringer. We concluded that exposure to steroids at this concentration does not alter transport of choline. This negative result is similar to most other drugs tested *in vitro*, except HC-3, tricyclic antidepressants, and choline analogues (18, 19).

High-affinity choline accumulation in synaptosomes from cats which received daily adrenocorticoid treatment for one week. We compared the effect of various steroid or vehicle treatments on HACA in the CP (Table 3). Each vehicle used to deliver steroids was assessed both after being given daily for one week or given 3 or 24 hours before sacrifice. Vehicles included peanut oil, *Aristocort* vehicle, and *Hydrocortone* vehicle; cats were given a volume proportional to body weight (1–1.5 ml/kg). Pharmacologically suspect components in the commercial vehicles included benzyl alcohol (0.9%) and Tween 80 (<0.8%). In addition, we tested the effect of 11α-epicortisol [11α,17α,21-trihydroxy-4-pregnene-

3,20-dione], a biologically-inert epimer of hydrocortisone given daily for one week at 8 mg/kg.

When HACA in untreated, vehicle-injected and 11α-epicortisol-treated cats were compared in CP synaptosomes (Table 3), no significant differences were found ($p > 0.05$, Kruskal-Wallis Test). Therefore, we concluded that the components of the vehicle, the stress of handling and injection, or pretreatment with a hydrocortisone epimer devoid of glucocorticoid action do not alter HACA in brain synaptosomes.

The results of intensive daily treatment with adrenocorticoids for one week on *in vitro* HACA in brain synaptosomes is presented in Table 3. The effect of two glucocorticoids (triamcinolone, 8 mg/kg; hydrocortisone, 4 and 32 mg/kg) and one mineralocorticoid (deoxycorticosterone acetate 32 mg/kg) were assessed in each tissue. Only results in the CP were consistently significant.

Throughout each of these chronic experiments, a few cats did not show an increase in CP HACA at the end of a full-week of treatment. The percentage of "non-responding" animals (defined as those with less than a 10% increase in HACA) ranged from 12% (1 of 8) in the DOCA treatment group to 25% (3 of 12) in each hydrocortisone group (see Table 3). Mongrel cats are likely to have a higher genetic variation in drug metabolism than inbred strains of rodents. Such genetic variation could, in certain animals, result in: a) a predisposition

TABLE 3

Regional effects of chronic gluco- or mineralocorticoid treatment on high-affinity accumulation of [^{3}H]choline in cat forebrain synaptosomes

Rates of [^{3}H]choline accumulation are expressed as pmoles/mg protein/4 min ± SEM. Numbers in parentheses are coefficients of variation (%).

Treatment	n	Controls	n	Treated	% Increase
Caudate-putamen					
Hydrocortisone, 4 mg/kg	14	8.31 ± 0.44 (20)	12	11.42 ± 0.87 (26)	+37**
Hydrocortisone, 32 mg/kg	19	7.71 ± 0.41 (23)	12	12.63 ± 0.90 (25)	+64**
Triamcinolone, 8 mg/kg	19	8.26 ± 0.36 (19)	6	14.29 ± 2.21 (38)	+73*
DOCA[a], 32 mg/kg	19	8.26 ± 0.36 (19)	8	11.96 ± 0.93 (22)	+45**
11α-epicortisol, 8 mg/kg	18	7.85 ± 0.44 (24)	2	6.91 ± 0.20 (4)	−12 NS
Vehicle-injected[b], 1-1.5 ml/kg	18	7.85 ± 0.44 (24)	13	7.18 ± 0.65 (33)	−8 NS
Anterior perforated space					
Hydrocortisone, 32 mg/kg	5	6.65 ± 1.38 (46)	5	8.46 ± 0.41 (11)	+27 NS
Triamcinolone, 8 mg/kg	9	9.26 ± 0.65 (21)	2	7.64 ± 0.88 (16)	−17 NS
DOCA, 32 mg/kg	9	9.26 ± 0.65 (21)	2	8.78 ± 0.64 (10)	−5 NS
Hippocampus-fornix					
Hydrocortisone, 4 mg/kg	9	5.86 ± 0.37 (19)	12	4.95 ± 0.27 (19)	−16*
Hydrocortisone, 32 mg/kg	14	4.64 ± 0.52 (42)	11	3.71 ± 0.35 (32)	−20 NS
Triamcinolone, 8 mg/kg	11	5.56 ± 0.36 (22)	6	4.72 ± 0.70 (36)	−15 NS
DOCA, 32 mg/kg	11	5.56 ± 0.42 (25)	8	6.39 ± 1.03 (46)	+17 NS
Prefrontal neocortex					
Hydrocortisone, 4 mg/kg	13	2.13 ± 0.14 (23)	4	2.56 ± 0.26 (20)	+20 NS
Hydrocortisone, 32 mg/kg	18	1.90 ± 0.16 (36)	8	1.76 ± 0.42 (68)	−7 NS
Triamcinolone, 8 mg/kg	13	2.13 ± 0.14 (23)	5	2.50 ± 0.34 (30)	+17 NS
DOCA, 32 mg/kg	13	2.13 ± 0.14 (23)	4	2.62 ± 0.24 (18)	+23 NS

[a] Deoxycorticosterone acetate.

[b] Vehicle-injected animals include: peanut oil, daily, seven days ($n = 2$); *Aristocort* vehicle, daily, seven days ($n = 2$); *Hydrocortone acetate* vehicle, single injection, 24 hours before sacrifice ($n = 2$); *Hydrocortone acetate* vehicle, single injection, three hours before sacrifice ($n = 2$); *Hydrocortone acetate*, daily for seven days ($n = 5$). Injection vehicles included at least benzyl alcohol (0.9%) and Tween 80 (<0.8%).

* $p < 0.02$.

** $p < 0.002$.

NS = $p > 0.05$, Mann-Whitney U-test, two-tailed.

to inactivate quickly an increased circulating level of steroid; or b) a capacity to accelerate inactivation, perhaps by enzyme induction, after initial high-level exposure to steroid. These findings suggest that the plasma corticoid level produced during daily depot treatment was not sufficiently high to overwhelm the homeostatic capacity of steroid regulatory systems in some (12–25%) of the treated animals.

In the CP, all the adrenocorticoid treatments produced a significant increase in HACA (assayed at 0.04 μM choline) compared with appropriate controls ($p < 0.02$, triamcinolone; $p < 0.002$, all others; Mann-Whitney U-Test, two-tailed). A dose-related trend exists within the two hydrocortisone treatment groups and the control group. If individual accumulation rates in these three groups are assumed to be in ascending order (H_1: 0 mg/kg < 4 mg/kg < 32 mg/kg; $n = 19; 12; 12$) a significant dose-related trend can be detected, with a strong Kendall rank coefficient ($\tau = 0.68$), significantly different from zero ($p < 0.0001$, Joncheree's Test for An Ordered Alternative).

In contrast, HACA in the HF is *not* increased by glucocorticoid treatment (Table 3). Hippocampal values were often 10–20% lower than controls—although only in a group with low variance (4 mg/kg hydrocortisone) was this effect significant ($p < 0.02$, Mann-Whitney U-Test, two-tailed). A 20% increase in HACA of the mineralocorticoid treatment group (DOCA, 32 mg/kg) was not significant compared to controls or other treatment groups ($p > 0.05$, Student-Newman-Keuls Test).

In the APS and the PFC, no significant differences emerged with treatment. However, in individual experiments the PFC showed a tendency to increase 15–25%, which was not apparent in the APS.

Mineralocorticoid treatment with a high-dose of DOCA (32 mg/kg) significantly increased HACA in the CP and produced small, insignificant rises in the APS and HF. Comparing mineralocorticoid and glucocorticoid-induced changes after equal doses, the overall percent increases caused by glucocorticoid were greater in the APS (+32%) and CP (+19%). In contrast, the glucocorticoid-induced response was smaller than the mineralocorticoid response in the HF (−37%) and PFC (−30%).

Effect of adrenocorticoids on [^{3}H]choline transport velocity and affinity. We investigated the kinetics of [^{3}H]choline transport to ascertain whether steroids were increasing accumulation in synaptosomes by an increase in uptake velocity, affinity, or both. We estimated the V_{max} and the K_T by Eadie-Hofstee plots (*e.g.*, Fig. 1) over a choline concentration range of 0.04 μM–2.5 μM. Since our purpose was to understand how steroid treatment increased accumulation of [^{3}H]choline in the CP, we examined data from those cats that responded to treatment with a greater than 10% increase in HACA. These are referred to below and in Tables 4 and 5 as "responders." In the HF, a tissue in which no significant change in HACA (assayed at 0.04 μM choline) was detected, we present data from all animals. Table 4 presents data from animals treated with low (4 mg/kg hydrocortisone) and high (8 mg/kg triamcinolone) daily doses of glucocorticoid or a high dose of mineralocorticoid (32 mg/kg DOCA).

In the CP, all three treatments produced a significant increase in the V_{max} (+45 to +53%) as compared with control values ($p < 0.01$, Student-Newman-Keuls Test), but not to each other. However, the K_T of CP synaptosomes remained unchanged, except after triamcinolone (8 mg/kg) treatment. In this case, the K_T decreased 22%. These treatment differences cannot be explained by changes in endogenous (extravesicular) choline levels, which may have been caused by steroid treatment. The isotopic dilution that would follow diffusion of this endogenous choline would have affected the observed K_T for HACA (20). This was not the case (Table 4). Thus, the cause of increased choline accumulation in CP synaptosomes is explained by an increase in the V_{max}.

In the HF, no significant changes were found in the V_{max} after any steroid treatment ($p > 0.05$, Student-Newman-Keuls Test). Moreover, the K_T for choline was unchanged, except after treatment with triamcinolone (8 mg/kg). Triamcinolone treatment caused an 84% increase in the

TABLE 4

V_{max} and apparent K_T of high-affinity [^{3}H]choline accumulation in untreated and adrenocorticoid-treated cats

V_{max} and apparent K_T estimated by Eadie-Hofstee plots with external choline concentration between 0.04–2.5 μM at 37°. V_{max} is expressed as mean pmoles/mg protein/4 min ± SEM. K_T is expressed as mean micromolar choline concentration ± SEM. Numbers in parentheses are coefficients of variation (%).

Treatment	*n*	V_{max}	% Incr.	K_T	% Incr.
Caudate-putamen[a]					
Untreated	6	124 ± 10 (20)	—	0.63 ± 0.03 (11)	— NS
Hydrocortisone (4 mg/kg)	4	182 ± 18 (20)	+47**	0.61 ± 0.01 (3)	−3 NS
Triamcinolone (8 mg/kg)	3	180 ± 13 (12)	+45**	0.49 ± 0.04 (15)	−22*
DOCA (32 mg/kg)	2	190 ± 7 (5)	+53**	0.65 ± 0.05 (11)	+3 NS
Hippocampus-fornix[b]					
Untreated	7	58 ± 4 (21)	— NS	0.44 ± 0.02 (14)	— NS
Hydrocortisone (4 mg/kg)	4	62 ± 6 (21)	+7 NS	0.52 ± 0.06 (22)	+18 NS
Triamcinolone (8 mg/kg)	3	79 ± 12 (25)	+36 NS	0.81 ± 0.13 (27)	+84*
DOCA (32 mg/kg)	2	88 ± 21 (34)	+52 NS	0.54 ± 0 (0)	+23 NS
Prefrontal Neocortex					
Untreated	6	24 ± 4 (44)	—	0.43 ± 0.09 (51)	—

[a] Only animals that responded to treatment with increased (>10%) accumulation are considered.

[b] All animals treated are considered.

NS–All pairwise comparisons of values marked NS are not significantly different from one another ($p > 0.05$).

* $p < 0.05$, Student-Newman-Keuls Test.

** $p < 0.01$, each value different from control but not significantly different from each other. Student-Newman-Keuls Multiple Test.

affinity of the transport system for [^{3}H]-choline. This dose of triamcinolone, equivalent to 32 mg/kg hydrocortisone, is capable of affecting synaptosomal transport affinity in the opposite direction of the CP and HF. In contrast, an 8-fold lower glucocorticoid dose (hydrocortisone, 4 mg/kg) produced a 60% increase in caudate V_{max} without affecting the K_T.

Thus, the transport of choline in the CP and HF is affected very differently by chronic adrenocorticoid treatment: a) HACA is increased in the CP, but not in the HF with hydrocortisone or DOCA; b) the V_{max} of caudate, but not hippocampal, transport is increased; c) the K_T of caudate synaptosomes is decreased by chronic treatment with triamcinolone (8 mg/kg).

Effect of hemicholinium-3 on HACA of synaptosomes from steroid-treated cats. HC-3 inhibition was assessed in CP and HF synaptosomes of cats treated with 32 mg/kg hydrocortisone for one week (Table 2). IC_{50} values from either CP or HF synaptosomes were similar to each other and indistinguishable from values from untreated cats. We conclude that the action of steroids on HACA does not affect the competitive inhibition of [^{3}H]choline transport by HC-3.

Effect of single intravenous dose of methylprednisolone on HACA. An acute dose of a soluble glucocorticoid might affect forebrain HACA. Therefore, we assessed the effect of methylprednisolone sodium succinate (90 mg/kg, *Solu-Medrol*) administered intravenously 3 or 24 hours before sacrifice. We compared this to vehicle-treated controls in the same experiment injected three hours before sacrifice to give a conservative estimate of response. Table 5 represents the change between vehicle and methylprednisolone-injected animals.

HACA in the CP responded to steroid treatment, showing a mean percent change of +35% at 3 hours and +56% at 24 hours (Table 5). The mean percent change in the APS was small at 3 hours (+7%), but increased at 24 hours (+44%). The remaining two tissues, the HF and PFC, had consistent, small changes of −9 to +24% at these times.[7] Yet, if only those animals that dem-

[7] Responses to intravenous methylprednisolone at 3 and 24 hours showed a large variability (coefficient of variation (CV) = 77–386%). The smallest variability

TABLE 5

Regional effects on high-affinity [^{3}H]choline accumulation after a single intravenous dose of methylprednisolone sodium succinate

Methylprednisolone sodium succinate (*Solu-Medrol*, Upjohn) was given intravenously (90 mg/kg) 3 or 24 hours before sacrifice to five animals. Values are mean percent change (%) in HACA compared to controls that received *Solu-Medrol* vehicle three hours before sacrifice in the same experiment.

Tissue	+ 3 hr		+ 24 hr	
	All	Rsp.[a]	All	Rsp.[a]
Caudate-putamen	+35	+75 ± 71 (3)	+56	+70 ± 32 (4)
Anterior perforated space	+7	+36 ± 4 (2)	+44	+76 ± 39 (3)
Hippocampus-fornix	+18	+84 ± 18 (2)	−9	+13 (1)
Prefrontal neocortex	+24	+40 ± 27 (4)	+20	+53 ± 23 (3)

[a] Rsp. = Responders: Mean percent change (%) ± SD of animals demonstrating a greater than 10% increase in HACA. Numbers in parentheses are the number of responders among the five animals treated.

onstrated a greater than 10% increase in HACA are considered (Table 5, "responders") at 3 hours, the CP and HF show twice the response (+75% and +84%) of the APS and PFC (+36% and +40%). However, by 24 hours the increase in the CP and APS is greater (+70% and +76%) than the HF and PFC (+13% and +53%).

From these results, it is clear that the initial response to acute methylprednisolone treatment is greatest in both the HF and the CP. This increase (75–84%) is equivalent in magnitude to responses in the CP after chronic treatment. By 24 hours, HACA in the APS has also reached this apparent treatment plateau, while the initial response in the HF has decreased to control levels. When these results after acute treatment are compared to responses after one week of chronic treatment, only the CP demonstrates an approximately +75% increase in HACA at all times. Thus, we conclude that the CP has a unique capacity for rapid and *sustained* increase of HACA. The HF and APS have distinct transitory capacities to increase, but not maintain, higher rates of HACA during periods of continued adrenocorticoid exposure.

Effect of high sodium and/or low potassium Krebs-Ringer on HACA. HACA in brain synaptosomes has been demonstrated to have strong ion dependency (Na^+/K^+); moreover, adrenocorticoids are able to alter electrolyte balance. Therefore, we undertook *in vitro* experiments to test the sensitivity of synaptosomes to altered Na^+/K^+ concentrations. We chose concentrations that approximate those of animals with steroid-induced hypernatremia and/or hypokalemia. Synaptosomes were prepared and pooled from three untreated cats and HACA assays conducted in Krebs-Ringer solutions modified as described in METHODS. Changes in HACA are presented in Table 6 with reference to synaptosomes assayed in isosmotic medium.

We examined HACA in synaptosomes from three brain regions, the CP, HF, and PFC. Raising the sodium concentration 30 or 60% produced changes in tissue HACA ranging between −8 and +17%; lowering the potassium concentration 30 or 60% produced changes between −13 and +9%. The presence of a strongly hypernatremic-hypokalemic Krebs-Ringer solution (Na^+, +60%/K^+, −60%), representative of severe steroid-induced electrolyte imbalance, did not produce synergistic increases in HACA. In the CP, the one tissue in which HACA was consistently elevated by *in vivo* steroid treatment, HACA was increased only from +5 to +17%.

We concluded that none of these *in vitro* electrolyte alterations produced significant increases in HACA. Moreover, the magnitude of these increases is not sufficient to explain the larger increases in HACA noted in the CP after *in vivo* adrenocorticoid treatment.

DISCUSSION

We have defined the properties of HACA in four regions of cat forebrain and exam-

was at 24 hours in the CP (CV = 77%) and in the APS (CV = 116%). The variability within the responder group was smaller (CV = 11–95%).

TABLE 6

Effect of high sodium and/or low potassium Krebs-Ringer solution on high-affinity [^{3}H]choline accumulation in forebrain synaptosomes

Single values reflect pooled tissue samples from three cats assayed in triplicate at 37° with duplicate 1° blanks. Percent increase in Na^+/K^+ concentrations above the normal incubation concentrations were chosen to mimic extreme electrolyte imbalance due to high circulating levels of adrenocorticoids. Percent increase in HACA is referenced to isosmotic controls produced by addition of sucrose.

Krebs-Ringer		Caudate-Putamen	Hippocampus	Prefrontal Neocortex
		(%)	(%)	(%)
Na^+	+30%	+5	0	−8
	+60%	+17	−1	+12
K^+	−30%	+9	−13	−8
	−60%	+7	−10	−11
Na^+ +60% K^+ −60%		+5	+19	−7

ined adrenocorticoid-induced alterations in HACA kinetic parameters (V_{max} and K_T). The HACA system present in synaptosomes from cat forebrain exhibits five criteria previously demonstrated for rat hippocampus: a) an apparent K_T below 4 μM; b) sensitivity to HC-3 inhibition ($K_i \simeq 2.5$ nM); c) Na^+/K^+ dependence; d) energy dependence; and e) a distribution coextensive with known cholinergic innervation.

Choline accumulation rates in the cat hippocampus at 0.04 μM choline generally accord with those reported for the rat (2, 3). The V_{max} of cat hippocampal HACA (58 pm/mg protein/4 min) is also similar to those reported for the rat (49–69 pm/mg protein/4 min). To confirm the interspecies similarity of HACA we assayed these four regions in the rat with our own method (0.04 μM choline at 37°) and obtained absolute values and rank order of accumulation similar to the cat (CP ≃ APS > HF > PFC; D. K. Riker and A. Sastre, unpublished observation). HACA rates for rat APS and PFC had not been previously reported. Assayed at 0.04 μM choline, the cat CP and APS exhibit identical accumulation rates, which are twice the rate of the HF and four-times that of the PFC. In a like manner, the V_{max} of the CP is double the V_{max} of the HF, and five times that of the PFC. On the other hand, the K_T is the same in the HF and PFC; however, the K_T of the CP is significantly higher.

Our most striking finding is the selective, large increase in HACA in the CP of cats treated chronically *in vivo* with gluco- or mineralocorticoids. This change may be explained by an increase in the uptake V_{max} without alteration in the K_T. The mechanism represented here by which *in vivo* adrenocorticoids raise HACA corresponds with that reported for other drugs found to increase HACA in the central nervous system after *in vivo* administration (3); in all cases the V_{max} is elevated. Accumulation of choline in the CP of some cats was maximally elevated as much as 70–80% as early as three hours after a single intravenous injection of methylprednisolone (90 mg/kg). Similar increases could not be produced *in vitro* by addition of steroids or hypernatremic-hypokalemic Krebs-Ringer medium.

In contrast to the CP, the HF reacts to chronic adrenocorticoid treatment by a 15–20% decrease in HACA. This effect is also seen between 3 and 24 hours after methylprednisolone treatment. Significant alterations in HACA of the APS or PFC were not found after chronic treatment. However, 24 hours after methylprednisolone some cats showed increased HACA comparable to the increases in the CP. Gluco- and mineralocorticoids evidently increase the rate of uptake, decrease the rate of release, or increase the number of choline transport sites in the CP without affecting the affinity of these sites for choline or HC-3.

The HACA response dissimilarity of the cat CP and HF to gluco- and mineralocorticoids is reminiscent of the differing drug responses found in these regions in the rat. Pentobarbital, a drug that reduces HACA in the rat hippocampus, does not affect HACA in the rat striatum (2, 3, 18). Pentylenetetrazol, the only drug reported to stimulate HACA *in vivo* to the same degree as adrenocorticoids, elevates rat hippocampal and neocortical HACA 45–75%, yet does not alter striatal HACA (3, 18, 21). *In vitro* depolarization with potassium (62 mM) increases rat hippocampal accumulation five times more than in striatum (21). In the cat, we find a HACA tissue selectivity dif-

ferent from those previously reported. Adrenocorticoids *in vivo* significantly increase CP HACA, yet HF HACA is unchanged. All of these results provide evidence of a qualitative difference in the pharmacology of the HACA systems of the mammalian hippocampus and striatum. These regional pharmacologic differences in cholinergic response may relate to documented differences in presynaptic mechanisms of cholinergic neurons (21, 22).

The tissue and plasma concentrations of steroids were not measured in the present study. However, some previous data suggest that our chronic depot treatments may not have produced plasma levels far in excess of ranges encountered physiologically. Stress alone can double or triple resting glucocorticoid levels in the rat, and has produced levels as high as 20–30 μg/100 ml plasma (23). In the rat a single intraperitoneal dose of 4 mg/kg hydrocortisone sodium succinate creates a maximal plasma concentration of 50 μg/100 ml 15 min after injection (23). However, catabolism of hydrocortisone injected intravenously in the cat or intraperitoneally in the rat reduces initial plasma levels 80–90% one hour after treatment (23). Therefore, the sequestration and presumed slower diffusion rate from an intramuscular site of 4 mg/kg hydrocortisone acetate given in a depot formulation probably does not maintain plasma glucocorticoid levels beyond the range produced by the physiological response to stress.

We can offer no mechanistic explanation for how adrenocorticoids affect HACA in CP. However, four possibilities merit discussion: a) a primary site of action affecting dopaminergic afferents to striatal cholinergic interneurons; b) alteration of the local ionic milieu; c) reduction in endogenous Ach levels; and d) a primary site of action on the cholinergic interneurons. We will briefly examine the evidence for these explanations.

First, glucocorticoids are known to increase accumulation of dopamine (D. K. Riker and R. Roth, unpublished observation), norepinephrine (8), and tryptophan (24) in brain synaptosomes or slices. Although most workers agree that synthesis-secretion of Ach is coupled to choline uptake in the HF and treatments that alter the level or turnover of Ach alter the uptake of choline, pharmacologic blockade of the dopamine input to the rat and avian striatum fails to alter HACA (21) (D. K. Riker, L. Choi and R. Roth, unpublished observation). Yet such blockade accelerates Ach turnover in striatum. Further, the cat APS, an area receiving meso-telencephalic dopamine input, and the septum and HF areas receiving extensive noradrenergic afferents from the locus coeruleus, do not respond to steroid treatment with enhanced HACA. Guyenet *et al.* make the point that the homeostatic regulation of Ach levels after pharmacological treatments that alter neuronal activity is not impressive in the striatum (25). These cited results suggest that striatal HACA may not be tightly coupled to transmitter release (at least that release that is modulated by afferent input). Our observation that gluco- and mineralocorticoids selectively elevate the V_{max} of HACA only in the striatum, a region in which HACA appears not to be coupled to transmitter release determined by afferent modulation, suggests that the response to steroids in this tissue may be independent of immediate transmitter demand. Consequently, these neurons may have the unique capacity to alter the maximal accumulation rate of choline in response to changes in the local concentration of hormonal substances that may not be immediately related to events at the nerve terminals.

Anatomical differences may underlie the differential sensitivity of CP cholinergic neurons to pharmacologic interventions. For example, cholinergic nerve terminals in the CP arise from intrinsic interneurons, whereas the cholinergic innervation of the HF is extrinsic. Furthermore, the predominant modulating monoamine in the CP is dopamine, whereas the HF and neocortex receive extensive noradrenergic input from the locus coeruleus.

Second, steroids are well-known modulators of Na^+/K^+ electrolytes (26) and HACA is Na^+/K^+ dependent (2, 3). However, striatal HACA could not be stimulated *in vitro* by hypernatremic-hypoka-

lemic medium. In addition, the regional tissue selectivity and the nearly equal responsiveness of the CP to gluco- or mineralocorticoids argue against any simple, nonspecific ion-mediated event. However, the Na^+/K^+ microenvironment of the striatum may participate in the *in vivo* increase of HACA.

Third, the steroid treatment may lower endogenous Ach levels; this could result in an increased HACA. Given the lability of Ach levels in the striatum (25) and the striatum's greater dependence on choline from Ach hydrolysis, this possibility remains.

The fourth mechanism, that adrenocorticoids directly affect HACA in the CP, seems most likely. McEwen and co-workers have demonstrated that glucocorticoids bind to cytoplasmic receptor proteins in brain (27). Although cytoplasmic receptors have not been identified in the CP (28), a recent study (29) reveals that glucocorticoids bind specifically to purified nerve terminal membranes. These membrane binding sites are characterized by different affinity and regional profile than those of cytosol. Thus steroids, entering certain nerve terminal membranes, may directly initiate or enhance neural activity. This appears to be the case with respect to the glucocorticoid enhancement of the post-tetanic responsiveness of the cholinergic motor nerve terminals in cat soleus muscle (6). Furthermore, the specificity of this nerve membrane response is emphasized by the recent finding that the cholinergic motor nerve terminals of cat gastrocnemius muscle are not similarly affected by this intensive glucocorticoid treatment (30). Such data not only disclose that nerve terminals within a neural system can differ pharmacologically, but also illustrate why regional differences may occur in the central nervous system. In view of this peripheral nervous system difference (30) and the reports of Sherman *et al.* (21) and Collier *et al.* (22), it may be that adrenocorticoids directly and selectively increase activity in the cholinergic interneurons of cat CP. This effect could be reflected by an increased HACA. Conversely, the lack of an adrenocorticoid effect on cat septal-hippocampal neurons may be accurately reflected by our finding of unchanged HACA in these neurons. If one assumes that the neural effects of intensive adrenocorticoid dosing represent a direct action on nerve membrane, the regional differences and the lack of effectiveness of 11α-epicortisol described here strongly suggest that specific molecular requirements determine this membrane action.

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