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Effects of Cycloheximide, a Protein Synthesis Inhibitor, on Mouse Brain Catecholamine Biochemistry[1]

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FREEDMAN, L. S., M. E. JUDGE AND D. QUARTERMAIN. *Effects of cycloheximide, a protein synthesis inhibitor, on mouse brain catecholamine biochemistry.* PHARMAC. BIOCHEM. BEHAV. 17(2) 187–191, 1982.—Cycloheximide (CXM), a protein synthesis inhibitor, has been shown to result in a marked inhibition of central catecholamine (CA) synthetic mechanisms at doses that cause amnesia in animals. Unlike other inhibitors of CA synthesis no significant depletion of whole brain NE or DA concentrations was observed 0.75, 1, 2, 3, 4, 6, 17, or 24 hours after administration of CXM (120 mg/kg) to C57BL/6J mice. In order to investigate the underlying basis of maintenance of CA levels in face of CA synthesis inhibition, the effects of CXM on *in vitro* release of ^{3}H-NE was studied in mouse hypothalamic slices. CXM, in a dose related manner, significantly inhibited the potassium stimulated release of NE from hypothalamic slices. Anisomycin, another protein synthesis inhibitor, similarly inhibited NE release. These studies further document the effects of protein synthesis inhibitors on CA mechanisms and suggest that disruption of CA biochemistry may play a role in the amnesia observed after administration of protein synthesis inhibitors.

Protein synthesis inhibitors Cycloheximide Anisomycin Norepinephrine and dopamine levels Norepinephrine release Amnesia

OVER the past 15 years, much research has utilized protein synthesis inhibitors (PSI's) as experimental tools to study the physiological and biochemical mechanisms involved in memory processing (for review see [2]). The results of this research have been interpreted as indicating that (a) the inhibitors are inducing amnesia solely because of their effect on protein synthesis and (b) protein synthesis is necessary for the formation of long term memory (e.g., [1,23]). This conclusion has been challenged by findings which indicate that PSI's also have complex side effects on central catecholamine (CA) systems. It has been shown that PSI's can modify CA activity by inhibiting central CA synthesis [8,13] and by inhibiting tyrosine hydroxylase activity *in vitro* [7,24]. Additional evidence consistent with the view that CA's may be involved in amnesias induced by PSI's comes from studies which show (a) that these amnesias can be attenuated by agents which activate pre- and post-synaptic CA mechanisms [4, 17, 18, 19, 21] and (b) that amnesias induced by inhibition of protein and CA synthesis frequently have common behavioral characteristics [17,18]. Taken together, these data suggest that interference with CA activity may contribute to the production of the amnesia which follows treatment with PSI's.

Studies [3, 8, 13] of the effects of PSI's on CA activity have shown that although rates of accumulation of newly synthesized norepinephrine (NE) and dopamine (DA) are significantly inhibited following PSI treatment, steady state levels of these amines were relatively unchanged at those times sampled. Maintenance of normal levels of CA's in the face of synthesis inhibition is an apparent paradox which could be resolved if it could be shown that the PSI's also decrease neurotransmitter utilization. The purpose of this investigation was to gain additional information on the effects of PSI's on catecholaminergic biochemical systems. Experiments were designed (a) to confirm the maintenance of normal CA levels by documenting in detail the time course of the effects of the PSI cycloheximide (CXM) on NE and DA levels in whole brain and various regions and (b) to test the hypothesis that PSI's decrease CA utilization by examining the effects of CXM and anisomycin (ANI) on potassium-stimulated release of NE from hypothalamic slices.

EXPERIMENT 1: STEADY STATE LEVELS OF BRAIN NOREPINEPHRINE (NE) AND DOPAMINE (DA)

Previous studies have indicated that protein synthesis in-

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TABLE 1

WHOLE BRAIN NOREPINEPHRINE AND DOPAMINE CONCENTRATIONS: EFFECT OF CYCLOHEXIMIDE AND DIETHYLDITHIOCARBAMATE

Time after drug (hours)	Norepinephrine Concentration (Mean μg/g ± SEM) (N)			Dopamine Concentration (Mean μg/g ± SEM) (N)		
	Control (saline)	CXM	DEDTC*	Control (saline)	CXM	DEDTC
0.75	0.48±0.030 (14)	0.45±0.028 (15)	—	1.18±0.067 (15)	1.25±0.082 (14)	—
1.00	0.54±0.022 (4)	0.55±0.028 (4)	—	1.74±0.197 (4)	1.81±0.205 (4)	—
2.00	0.43±0.022 (12)	0.38±0.024 (13)	0.27±0.022 (13)	1.92±0.204 (12)	1.62±0.183 (13)	1.77±0.141 (13)
3.00	0.45±0.028 (3)	0.50±0.001 (4)	0.28±0.040 (3)	1.73±0.088 (5)	1.49±0.088 (6)	1.51±0.136 (3)
4.00	0.43±0.064 (6)	0.43±0.026 (6)	0.29±0.026 (6)	—	—	—
6.00	0.41±0.028 (18)	0.37±0.026 (16)	—	1.86±0.139 (19)	1.63±0.095 (21)	—
17.00	0.41±0.032 (15)	0.37±0.022 (15)	—	1.21±0.063 (16)	1.25±0.083 (15)	—
24.00	0.40±0.016 (20)	0.39±0.016 (20)	—	1.32±0.098 (7)	1.27±0.063 (7)	—

Number of animals per group shown in parentheses.
*$F(1,37)=24.05$, $p<0.01$: from comparison to control means (see text).
CXM administered at 120 mg/kg SC; DEDTC at 250 mg/kg SC.

hibitors paradoxically maintain normal steady state levels of catecholamines in the face of marked inhibition of CA synthesis [8,13]. This experiment was designed to more fully investigate regional and whole brain NE concentrations at various times after a dose of CXM which has been shown to induce amnesia [19]. For comparison purposes, CA levels were also measured after administration of diethyldithiocarbamate, a potent inhibitor of NE synthesis.

METHOD

C57BL/6J male mice, 20–25 g, were housed in groups of eight with food and water ad lib. Mice (N=211) were injected subcutaneously with either saline, 0.9%, cycloheximide, 120 mg/kg, or diethyldithiocarbamate (DEDTC), 250 mg/kg. We have previously demonstrated that these drug doses produce amnesia in mice [9]. CXM and Saline treated animals were killed by cervical dislocation 0.75, 1, 2, 3, 4, 6, 17 and 24 hours after drug administration. DEDTC injected mice were killed 2, 3, and 4 hours after drug. Brains were rapidly removed and dissected on ice [11]. For catecholamine determination, tissue was weighed, then homogenized in 0.4 N perchloric acid. Catecholamines were isolated by alumina chromatography [22] and assayed by spectrofluorometric techniques [16]. Data from the CXM and Saline groups were analyzed by 2-way analyses of variance. Comparisons between group means were performed using Student's *t*-test.

RESULTS

The results of this experiment are shown in Table 1. Two-way analyses of variance indicated that CXM, 120 mg/kg, did not alter NE or DA concentrations in the brain 0.75, 1, 2, 3, 4, 6, 17, or 24 hours after drug administration. There was no significant overall difference between CXM and Saline groups: NE; $F(1,169)=0.89$, DA; $F(1,144)=1.26$, and no significant interaction of drug treatment with time: NE; $F(7,169)=0.44$, DA; $F(6,144)=0.563$. For comparison purposes, NE and DA levels were measured after administration of 250 mg/kg, DEDTC, a drug that blocks the synthesis of NE from DA by inhibiting the enzyme dopamine-beta hydroxylase [12]. Consistent with previous data from several laboratories this agent caused a significant depletion of brain NE 2, 3, and 4 hours after administration, (compared to appropriate control values: $F(1,37)=24.05$, $p<0.01$), but at 2 and 3 hours there was no change in DA levels, $F(1,29)=0.65$.

The effects of CXM on regional levels of NE are shown in Table 2. CXM results in an acute rise of 12.5% ($t=3.45$, $df=8$, $p<0.01$) in brain stem NE but no change in NE concentration in cortex or hypothalamus. Thus, despite the presence of an agent known to inhibit brain CA synthesis, levels of NE and DA were maintained at normal concentrations, and in one instance (brain stem, 1.5 hrs) NE levels actually increased.

EXPERIMENT 2: *IN VITRO* RELEASE OF ^{3}H-NOREPINEPHRINE FROM HYPOTHALAMIC SLICES

Decreased utilization of catecholamines would explain the phenomenon of maintenance of normal steady state levels of CA's in face of CA synthesis inhibition. This experiment was designed to examine whether a defect in central NE release mechanisms could be demonstrated. To investigate the status of NE release mechanisms, ^{3}H-NE release was measured in hypothalamic slices using a modified superfusion technique [25]. To obtain an index of the functional status of release mechanisms the effect of CXM on the stimulatory effect of potassium induced depolarization was measured. The hypothalamus was selected as representative of an area particularly rich in NE terminals.

METHOD

C57BL/6J mice (N=87) were sacrificed as previously described and hypothalamic slices (300 μM thick, rostrocaudal plane) were obtained from fresh tissue using a McIlwain tissue slicer. For each determination, slices from three mice pooled (18–24 slices total), placed in oxygenated (95% O_2: 5% CO_2) Krebs-Ringer bicarbonate (KRB) medium and incubated for 10 minutes with 0.1 μM ^{3}H-NE (S.A. 10.43 Ci/mmol, NE Nuclear). Subsequently, radio-labelled slices were superfused at a rate of 1.0 ml/minute for thirty minutes

TABLE 2

REGIONAL LEVELS OF NOREPINEPHRINE: EFFECT OF CYCLOHEXIMIDE
NOREPINEPHRINE CONCENTRATION (MEAN μg/g ± SEM (N))

Time After Drug (hours)	Group	Brain Region		
		Cortex	Brain Stem	Hypothalamus
1.5	Control	0.53 ± 0.044 (6)	0.72 ± 0.014 (5)	1.37 ± 0.081 (6)
	CXM	0.48 ± 0.031 (6)	0.81 ± 0.022* (5)	1.40 ± 1.08 (5)
3.0	Control	0.44 ± 0.037 (6)	0.64 ± 0.047 (10)	1.96 ± 0.251 (8)
	CXM	0.39 ± 0.028 (6)	0.63 ± 0.054 (10)	1.48 ± 0.162 (9)

Number of animals per group shown in parentheses.
CXM administered at 120 mg/kg SC.
*$p<0.01$: from comparison to control mean (see text).

[25]. The basal efflux of radioactivity initially was high due to label in extracellular spaces, then dropped quickly during the first thirty-minutes, reaching a fairly stable baseline. Depolarization induced release was then produced by a one minute pulse of superfusion with a KRB medium containing 40 mM K^+. Fractions were collected over two minute intervals during the following twenty minute superfusion period and the slices were homogenized in distilled water.

Exposure of slices to high K^+ medium, as expected resulted in a sharp increase in efflux of radioactivity which peaked within 2–4 minutes and returned to stable baseline by end of 20 minute period. Aliquots of fractions and tissue homogenates were analyzed for radioactivity by liquid scintillation spectrophotometry. In cycloheximide and anisomycin experiments, drug was present in the KRB medium at concentrations of $10^{-4} \times 10^{-2}$ M throughout basal and K^+ stimulated periods. Results were expressed as percent stimulated release calculated by the formula: $C-B/A \times 100$ where:

A is the total radiolabel in slices at the onset of K^+ stimulated release (KSR) obtained by summing radiolabel in all fractions from introduction of high K^+ medium together with that remaining in slices at end of superfusion;

B is average basal release of label during KSR period obtained by averaging basal release rates for (a) the last fraction before the high K^+ KRB pulse and (b) the first fraction after release returns to baseline; and

C is total label released during KSR (sum of radioactivity in all fractions from onset of high K^+).

Data from the release experiment were analyzed using the Student's t-test for comparison of mean values of appropriate groups.

RESULTS

The results of this experiment are shown in Fig. 1.

CXM resulted in a dose dependent reduction (57–65%) in potassium (40 mM) stimulated release. At a concentration of 10^{-4} M, CXM and control release rates were comparable, whereas at 10^{-2} and 10^{-3} M, release was significantly inhibited (respectively, (2-tail) $t(14)=4.03$, $p<0.002$; $t(10)=2.36$, $p<0.05$. Although the effective doses of CXM were relatively high, the demonstration of a response of cells to K^+ stimulation, albeit at a reduced magnitude, suggests that these results are not simply due to general cytotoxic effects of CXM. In fact, slices treated with CXM were able to respond to a second pulse of high K^+.

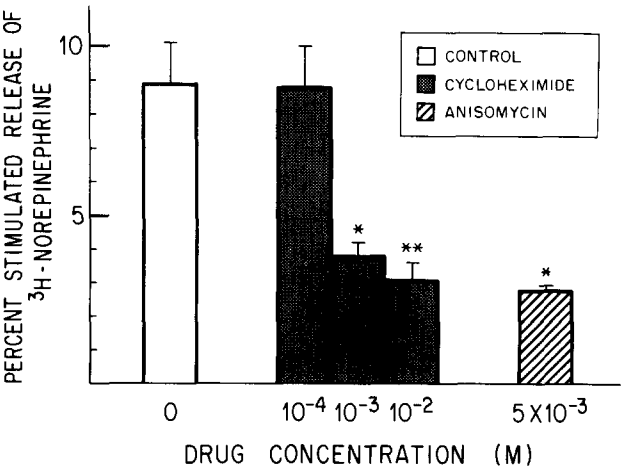

FIG. 1. Percent stimulated release of ^{3}H-norepinephrine from mouse hypothalamic slices. Results expressed as mean±S.E.M. *$p<0.05$ **$p<0.002$: from comparison to control mean (see text). Number of experiments: *control:* 9, *CXM:* 10^{-4} M: 8, 10^{-3} M: 3, 10^{-2} M: 7; *Ani:* 5×10^{-3} M: 2.

In order to ascertain whether the observed CXM induced inhibition of ^{3}H-NE release was idiosyncratic to CXM, the effect of the protein synthesis inhibitor anisomycin was also measured. Anisomycin, 5×10^{-3} M, caused a 69.7%, $t(9)=2.30$, $p<0.05$, suppression of K^+ stimulated release of ^{3}H-NE (Fig. 1). This observed reduction by both CXM and anisomycin suggests that inhibition of NE release may be a general property of agents that inhibit protein synthesis.

GENERAL DISCUSSION

The results of this study further document the effects of protein synthesis inhibitors (PSI's) on brain catecholamine (CA) biochemistry by (a) confirming that steady-state levels of NE are maintained after CXM treatments in spite of marked inhibition of CA synthesis, and (b) by demonstrating that exposure to CXM or anisomycin reduces the magnitude of potassium stimulated release of NE. CXM, at a dose that inhibits brain protein synthesis by 85–90% and results in amnesia, has also been shown to significantly inhibit the synthesis of brain catecholamines [8,13]. This CA synthesis in-

hibition may be related to the observed reduction of *in vitro* activity of tyrosine hydroxylase [7,24], the enzyme catalyzing the first step in the biosynthesis of dopamine (DA) and norepinephrine (NE). In the face of this inhibition, brain DA and NE levels do not exhibit the depletion exhibited after more classical inhibitors of DA and/or NE synthesis such as alpha-methyl-para-tyrosine and DEDTC [13]. In fact, Goodman *et al.* [13] have reported an acute rise in whole brain levels. We do not confirm this finding although in regional studies there was a significant rise in brain stem NE 1.5 hours after CXM. A situation of synthesis inhibition without a change in CA concentration could be accounted for by reduced neurotransmitter utilization, specifically CXM induced inhibition of release mechanisms. The finding that CXM *in vitro* is capable of reducing potassium stimulated release of hypothalamic NE is consistent with the hypothesis of a defect in NE release. The change in release induced by CXM is also observed with anisomycin, suggesting that the effects are not idiosyncratic to CXM and may be generalizable to protein synthesis inhibitors as a class. However, it should be noted that although we have demonstrated impaired release *in vitro* at relatively high concentrations of drug, there exists to date no direct evidence of impaired NE release *in vivo*.

That the observed action of CXM on CA presynaptic mechanisms may also involve postsynaptic changes is suggested by preliminary data from our laboratory that indicates that CXM *in vivo* and *in vitro* reduces the magnitude of NE stimulation of hypothalamic adenylate cyclase activity. Such a finding may be indicative of reduced responsivity of postsynaptic NE receptors.

The findings of this study contribute additional biochemical evidence that the mechanism of action of PSI's such as CXM involve not only the inhibition of the incorporation of amino acids into protein, but also profound disruption of various aspects of catecholamine mechanisms. Thus, protein synthesis inhibitors which are used to produce amnesias are capable of significantly "tuning down" central CA mechanisms. The demonstration that agents capable of inducing amnesia when administered at the critical time of information input also produce profound changes in the activity of neurotransmitter systems suggests that amnesias induced by PSI's may not be exclusively the result of their disruption of protein synthesis. There is ample evidence to involve CA's in memory processes. Pharmacological agents that disrupt CA systems by blocking synthesis [10,17], disrupting vesicular storage [6,15], or blocking receptors [5,14] are capable of inducing amnesia. Conversely, agents that activate CA's by pre- or post-synaptic mechanisms can reverse amnesia [6, 9, 19] and facilitate the retrieval of a partially forgotten habit [20]. These data are consistent with the view that CA's are involved in both the storage and the expression of learned behavior. Therefore we suggest that the induced disruption of CA mechanisms at the critical time of information input and processing should be considered as playing a significant role in the subsequent development of amnesia.

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