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Biochemical Actions of Sympathomimetic Drugs Which Overcome Cycloheximide-Induced Amnesia

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JEFFREY, P. L. AND M. E. GIBBS. *Biochemical actions of sympathomimetic drugs which overcome cycloheximide-induced amnesia.* PHARMAC. BIOCHEM. BEHAV. 5(5) 571–575, 1976. – Earlier investigations of sympathomimetic drugs overcoming the amnesic action of cycloheximide (CXM) in day-old chickens were extended to biochemical studies in vitro. The effects of amphetamine, norepinephrine, α and β noradrenergic stimulants and receptor blockers on Na^+/K^+ ATP'ase activity in total homogenate of chicken forebrain were investigated. Norepinephrine and the β stimulant, isoprenaline significantly stimulated the activity of this enzyme, while the β blocker, propranolol inhibited activity. Amphetamine, the α stimulant, methoxamine and the α receptor blocker, piperoxane had no effect on Na^+/K^+ ATP'ase activity in total homogenate. In a purified synaptosomal preparation, both amphetamine (5×10^{-5} M) and norepinephrine (1×10^{-4} M) produced a slight stimulation of Na^+/K^+ ATP'ase activity. A similar concentration of amphetamine (1.12×10^{-4} M) did not inhibit ^{14}C-leucine uptake or incorporation into protein in the synaptosomal fraction. Nor was it able to alleviate CXM inhibition of ^{14}C-leucine incorporation into synaptosomal protein. The results are interpreted in terms of amphetamine (via release of norepinephrine) norepinephrine and isoprenaline stimulating and maintaining the labile, sodium pump-dependent, phase of memory formation for a sufficient length of time until protein synthesis inhibition by CXM wears off.

Amphetamine Norepinephrine α and β noradrenergic stimulants and receptor blockers Day-old chickens
Labile protein synthesis-independent memory Permanent protein synthesis-dependent memory

RETENTION of memory for one-trial passive avoidance training in day-old chickens is high for at least 72 hr after learning [12]. Three phases have been isolated in the formation of the long-term memory storage [9]. The first, short-term, memory phase lasts for no longer than 10 min, and is not interfered with by the sodium pump inhibitor ouabain which blocks the succeeding labile memory phase. The labile phase is present for 30 min and is followed by a protein synthesis-dependent, long-term storage which can be blocked by the protein synthesis inhibitor cycloheximide (CXM). Under the influence of CXM the retention of the learning task starts to decrease after 30 min and has almost completely disappeared by 3 hr; with ouabain administered before learning the retention starts declining at 10 min and is also gone by 3 hr. The labile phase is necessary for the development of protein synthesis-dependent long-term memory [7, 12, 19].

Normally, when CXM is administered 5 min before the learning trial, there is very little memory left 3 hr later. However, if amphetamine [5] or norepinephrine [6] is administered subcutaneously soon after learning the amnesia produced by CXM does not occur and retention at 3 hr is normal. The time at which amphetamine or norepinephrine is administered is important. To overcome CXM-induced amnesia the drugs have to be administered while the labile, sodium pump-dependent memory is still present, i.e. earlier than 30 min after learning. At 120 min when labile memory has decayed, the administration of these drugs does not prevent CXM-induced amnesia. The level of retention at 3 or 24 hr after administration between 30 and 120 min after learning, depends on the memory left in the decaying labile store at the time of administration of the drugs.

How do amphetamine and norepinephrine overcome the amnesia produced by CXM? The behavioural results suggest that this action of amphetamine is indirect and is due partly to the release of norepinephrine, since amphetamine's action in overcoming CXM-induced amnesia can be stopped by blocking noradrenergic α and β receptors with the antagonists piperoxane and propranolol respectively. The

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blockers of serotonin, dopamine and histamine receptors have no effect on the action of amphetamine. The α and β noradrenergic stimulants, methoxamine and isoprenaline respectively, have the same effect as norepinephrine and amphetamine [6].

In terms of the three phase model of memory formation there are two phases where amphetamine or norepinephrine could exert their action in overcoming CXM-induced amnesia. They may exert their effects during the protein synthesis phase by either directly stimulating protein synthesis or preventing inhibition of protein synthesis by CXM through antagonistic effects on CXM itself. Alternatively they may act during the sodium pump phase through stimulation of Na^+/K^+ ATP'ase activity, possibly resulting either in increased amino acid uptake for specific protein synthesis or in prolonging Na^+/K^+ ATP'ase activity until protein synthesis recovers from the inhibitory effects of CXM.

To investigate these possibilities, the effects of amphetamine and norepinephrine on a number of biochemical systems of chicken forebrain, investigated in detail previously [8], were examined. The systems studied were Na^+/K^+ ATP'ase activity in both total homogenate and the synaptosomal fraction, incorporation of radioactive leucine into protein and its inhibition by CXM, and radioactive leucine uptake into the synaptosomal fraction.

METHOD

Animals

Day-old white-Leghorn black-Australorp cockerels, obtained from a local hatchery, were used throughout these experiments.

Procedure

Preparation of synaptosomal fraction from chick forebrain. All manipulations were carried out at 4°C. A 10% (w/v) homogenate of chick forebrain in 0.32 M sucrose containing 5 mM Tris (pH 7.4) was prepared using a glass/Teflon homogenizer (clearance 0.15 mm). Crude nuclear fractions were removed by two centrifugations at 1000 g_{av} for 10 min and one at 1500 g_{av} for 15 min. A crude mitochondrial pellet was obtained by centrifuging the supernatant at 11,500 g_{av} for 20 min. This pellet was washed three times, resuspended in the homogenizing medium, and layered onto a discontinuous gradient of 7.5% and 13% (w/v) Ficoll and 0.32 M sucrose and 5 mM Tris (pH 7.4). After centrifugation for 1 hr at 51,500 g_{av}, the synaptosomal fraction was removed from the interface of the 7.5 and 13% Ficoll layers, diluted four-fold with sucrose, and centrifuged at 80,000 g_{av} for 30 min. The synaptosomal fraction was suspended in the required medium before use.

Measurement of C^{14}-leucine incorporation into synaptosomal fraction. The obtained synaptosomal fraction was suspended in medium TMN of Morgan and Austin [13] containing 125 mM NaCl, 25 mM KCl, 15 mM $MgCl_2$, and 10 mM TRIS pH 7.4, at an approximate protein concentration of 0.5 mg/ml medium. The reaction was commenced by addition of 0.5μC_i of L-(U-^{14}C)- leucine (Amersham 312 mC_i/m mole) to 1 ml of incubation medium after 10 min preincubation at 37°C with or without added drugs. The reaction time was 30 min. Reactions were terminated by addition of an equal volume of cold 10% w/v TCA. Samples were prepared for counting in the manner described by Austin and Morgan [1]. The efficiency of the Triton scintillation counting procedure was 80%. Samples were taken for protein estimation by the method of Lowry *et al. [11].*

Estimation of free amino acid uptake into synaptosomal soluble pool. Using ^{14}C-leucine, the free amino acid uptake was determined in parallel with protein synthesis studies described by Morgan and Austin [13]. All incubations were for 30 min.

Preparation of chick forebrain homogenate and estimation of enzyme activity. Na^+/K^+ ATP'ase activity was measured in total homogenate prepared from chicken forebrain. After decapitation, the forebrain was removed, weighed and homogenized with 50 volumes of de-ionized distilled water after the method of Yoshimura [20]. The reaction medium contained in a final volume of 1 ml: brain homogenate (2.0 mg wet weight), 20 mM KCl, 120 mM NaCl, 7.5 mM $MgCl_2$, 5 mM ATP (Tris salt) and 30 mM Tris HCl buffer (pH 7.1). The reaction was carried out for 30 min and ATP'ase activity was measured by the amount of inorganic phosphate released, in the manner reported previously [8]. The Mg^{++} and Na^+/K^+ ATP'ase activities of synaptosomal fractions were determined as previously described [8]. The synaptosomal fraction was suspended in 0.32 M sucrose, 5 mM Tris HCl buffer (pH 7.4) before addition to incubations.

The effects of amphetamine, norepinephrine, ouabain, cycloheximide, methoxamine, piperoxane, isoprenaline and propranolol on the various parameters were carried out at the concentration reported in the various figures and tables to follow.

RESULTS

Effect of d-amphetamine and norepinephrine on Na^+/K^+ ATP'ase activity. Amphetamine at concentrations of between 10^{-5} and 10^{-3} M had no effect on the activity of Na^+/K^+ ATP'ase when measured in a forebrain total homogenate (Fig. 1). On the other hand, norepinephrine at concentrations greater than 10^{-4}M markedly increased Na^+/K^+ ATP'ase activity. Mg^{++} ATP'ase activity was not affected by any concentration of either amphetamine or norepinephrine.

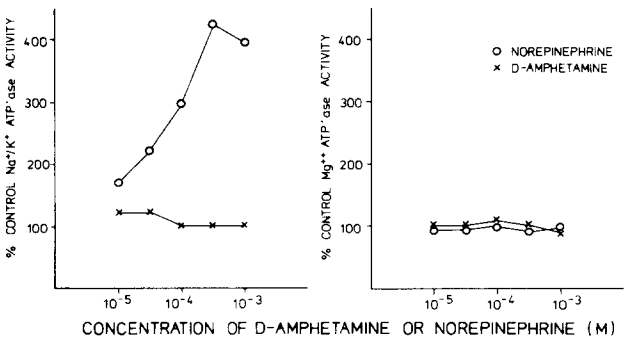

FIG. 1. The effect of norepinephrine and amphetamine on Mg^{++} and Na^+/K^+ ATP'ase activities from chicken forebrain total homogenate. Control activities for Mg^{++} ATP'ase with norepinephrine and amphetamine were 11.6 and 10.3 μmoles Pi liberated/ml extract/hour respectively. Control Na^+/K^+ ATP'ase activities for norepinephrine and amphetamine determinations were both 2.6 μmoles Pi liberated/ml extract/hour. Each ml of extract contained 2 mg wet weight protein. The result at each concentration is the average of 4 determinations and the values were within a range of 5% of the mean.

TABLE 1

THE EFFECT OF AMPHETAMINE AND NOREPINEPHRINE ON SYNAPTOSOMAL Mg^{++} AND Na^+/K^+ ATP'ase ACTIVITIES

Amphetamine concentration (M)	ATP'ases μmoles Pi liberated/mg protein/hr Mg^{++}	Na^+/K^+
—	10.6	5.3
1×10^{-5}	10.6	6.6
5×10^{-5}	11.0	7.1
1×10^{-4}	11.2	5.6
5×10^{-4}	10.6	6.5
1×10^{-3}	10.4	6.3
1×10^{-2}	11.1	3.9
Norepinephrine concentration (M)		
1×10^{-5}	10.9	5.7
5×10^{-5}	12.0	5.4
1×10^{-4}	12.0	7.0
5×10^{-4}	12.0	6.7
1×10^{-3}	12.6	6.0
5×10^{-3}	12.7	6.7
1×10^{-2}	9.0	6.1

In the synaptosomal fraction, amphetamine at 5×10^{-5}M and norepinephrine at 10^{-4}M produced a slight stimulation of the Na^+/K^+ ATP'ase activity (35.8% and 33.9% respectively) (Table 1). A concentration of 10^{-2}M amphetamine was the only one which inhibited Na^+/K^+ ATP'ase activity.

As shown in Fig. 2, the α noradrenergic stimulant, methoxamine and the α blocker, piperoxane, had no effect on either the Mg^{++} or the Na^+/K^+ ATP'ase activity in the total homogenate preparation. However, the β noradrenergic stimulant, isoprenaline, at concentrations greater than 5×10^{-5} M more than doubled the Na^+/K^+ ATP'ase activity while having no marked effect on the Mg^{++} ATP'ase activity. The β blocker propranolol, inhibited the Na^+/K^+ ATP'ase activity at concentrations greater than 10^{-4} M, with only a slight inhibition of Mg^{++} ATP'ase activity at 10^{-3}M.

Effect of d-amphetamine on incorporation and uptake of ^{14}C-leucine into the synaptosomal fraction. The incorporation of ^{14}C-leucine into protein by the synaptosomal fraction was not inhibited by concentrations of amphetamine up to 5×10^{-4}M, but higher concentrations produced an inhibition. At 5×10^{-3}M or 10^{-2}M amphetamine, the incorporation of leucine was inhibited by 50 to 60% (Table 2). This reduced incorporation was not due to an interference of protein synthetic mechanisms but rather due to reduced uptake of free ^{14}C-leucine, such that at amphetamine concentrations greater than 10^{-3}M, the uptake of ^{14}C-leucine was reduced by more than 50%. Amphetamine, cycloheximide and ouabain reduced the incorporation of ^{14}C-leucine into synaptosomal protein but only amphetamine and ouabain inhibited the uptake of ^{14}C-leucine into the synaptosomes.

When the synaptosomal fraction was incubated with CXM and amphetamine, it is apparent that amphetamine at any of the concentrations used did not overcome the inhibitory effect of CXM on the incorporation of ^{14}C-leucine into synaptosomal protein.

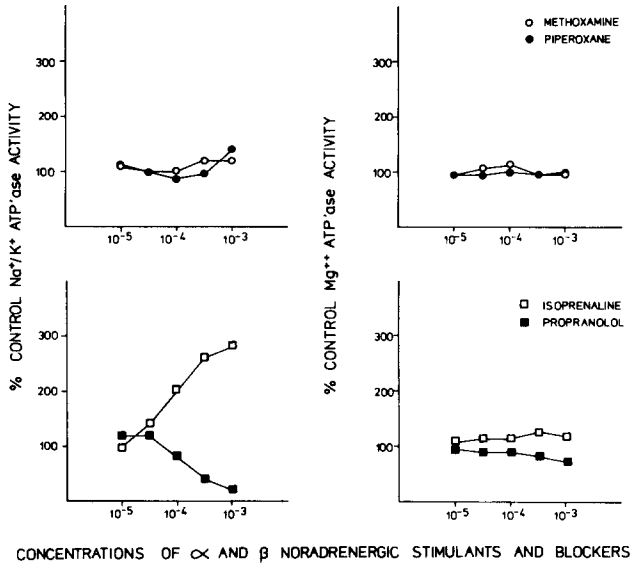

FIG. 2. The effect of the α agonist, methoxamine; the α antagonist, piperoxane; the β agonist, isoprenaline and the β antagonist, propranolol on Mg^{++} and Na^+/K^+ ATP'ase activities from chicken forebrain total homogenate. Control Mg^{++} ATP'ase activities were 11.0 ± 0.5 (S.D., 4 determinations) μmoles Pi liberated/ml extract/hr and for Na^+/K^+ ATP'ase activities 4.0 ± 1.0 (S.D., 4 determinations) μmoles Pi liberated/ml extract/hr. Each ml of extract contained 2 mg wet weight protein. The result at each concentration is the average of 4 determinations.

DISCUSSION

Amphetamine had no effect on Na^+/K^+ ATP'ase activity in a crude homogenate of chicken forebrain but produced slight stimulation of the activity of this enzyme in the synaptosomal fraction (35.8%) at a concentration of 5×10^{-5}M. However, norepinephrine (5×10^{-4}M) resulted in a four-fold increase in Na^+/K^+ ATP'ase activity in forebrain homogenate. At a similar concentration to amphetamine (10^{-4}M) norepinephrine increased Na^+/K^+ ATP'ase activity to the same extent as amphetamine in the synaptosomal fraction (33.9%). Yoshimura [20] has reported stimulation of Na^+/K^+ ATP'ase activity by norepinephrine in homogenate of areas of rat brain.

Amphetamine stimulates the release of norepinephrine [3] and also blocks the reuptake of norepinephrine [4, 14, 16]. Amphetamine administered *in vivo* has been reported to increase Na^+/K^+ ATP'ase activity in guinea pig brain [17]. The inability of amphetamine to stimulate Na^+/K^+ ATP'ase activity in the crude homogenate may be due to the fact that homogenization and treatment with distilled water will have released all the norepinephrine from the cells.

In the synaptosomal preparation, where the membrane bound structure remains intact, and certain key properties remain unaltered over extended periods [2], the observation that amphetamine can stimulate Na^+/K^+ ATP'ase activities to the same extent as norepinephrine supports the contention that amphetamine is releasing norepinephrine and the latter is responsible for the stimulation of the Na^+/K^+ ATP'ase activity.

These results indicate that the action of amphetamine and norepinephrine in overcoming CXM-induced amnesia may be due to stimulation of Na^+/K^+ ATP'ase activity

TABLE 2

EFFECTS OF AMPHETAMINE ON INCORPORATION AND UPTAKE OF ^{14}C-LEUCINE IN THE SYNAPTOSOMAL FRACTION

	Protein incorporation (dpm/mg)	Leucine uptake (dpm/mg x 10^3)	Protein incorporation in presence of 0.1mM cycloheximide
Control	370	51.2	(185)
Ouabain (1mM)	190	19.2	
CXM (0.1mM)	185	49.7	
D-Amphetamine (M)			
1.12×10^{-4}	323	46.1	155
5.56×10^{-4}	356	39.8	174
1.12×10^{-3}	239	46.2	169
5.56×10^{-3}	180	25.5	211
1×10^{-2}	157	14.2	162

^{14}C-leucine incorporation into protein and ^{14}C-leucine uptake by synaptosomes. The synaptosomal fraction was incubated for 30 min with ^{14}C-leucine and the incorporation and free amino acid pools determined as described in Method.

involved in holding the labile memory trace or to increased amino acid uptake for protein synthesis. This interpretation is supported by the action of the β noradrenergic drugs on Na^+/K^+ ATP'ase activity. The β stimulant, isoprenaline, which overcomes CXM-induced amnesia, markedly stimulated Na^+/K^+ ATP'ase activity, while the β receptor blocker, propranolol, which prevents amphetamine overcoming CXM-induced amnesia, inhibited Na^+/K^+ ATP'ase activity. The fact that α stimulants and blockers do not affect Na^+/K^+ ATP'ase activity while having the same behavioural effects as the β noradrenergic agents with respect to CXM-induced amnesia is not easily explained; although other α noradrenergic blocking agents have been reported to inhibit Na^+/K^+ ATP'ase activity in bovine brain [15].

The implications of the effects of low concentrations of amphetamine and norepinephrine on leucine uptake and protein synthesis in the synaptosomal fraction are not as yet clear. In these experiments higher concentrations of amphetamine (5×10^{-3}M) than those found to stimulate Na^+/K^+ ATP'ase activity (5×10^{-5}M) inhibit leucine incorporation into protein with 30 min of incubation, presumably by inhibiting uptake of the labelled amino acid. Norepinephrine (10^{-3}M) also has been reported to inhibit ^{14}C-leucine incorporation into protein over 30 min in a synaptosomal fraction from rat brain [10]. A lower concentration of norepinephrine (10^{-4}M) had no significant inhibitory effect on leucine incorporation. No evidence on the effects of this drug on amino acid uptake was reported. In this context, it may be noted that in the behavioural studies reported earlier [5] the blood concentration of amphetamine may be estimated to be of the order of 5×10^{-5}M, therefore, inhibition of protein synthesis is unlikely to be a factor in the behavioural experiments. It is also unlikely that amphetamine or norepinephrine would have a marked enough stimulation of ^{14}C-leucine incorporation into protein to overcome CXM-induced amnesia. In the present experiments, amphetamine at concentrations from 10^{-4} to 10^{-2} M did not have any effect in alleviating CXM-induced inhibition of leucine incorporation in the synaptosomal fraction, hence amphetamine does not appear to antagonize CXM inhibition of protein synthesis.

Amphetamine inhibits the reuptake of norepinephrine [4]. The present evidence suggests that it does not inhibit Na^+/K^+ ATP'ase. The sodium pump has been implicated in the reuptake of norepinephrine as ouabain inhibits both Na^+/K^+ ATP'ase activity and norepinephrine accumulation in synaptosomes [18]. However, Tissari *et al* [18] concluded that ouabain blocks the accumulation of norepinephrine by an indirect process resulting from inhibition of Na^+/K^+ ATP'ase, rather than directly. Therefore, it seems unlikely that amphetamine inhibits norepinephrine reuptake via Na^+/K^+ ATP'ase activity.

From the existing evidence it seems possible that the action of amphetamine in overcoming CXM-induced amnesia is due to increased Na^+/K^+ ATP'ase activity through the release of norepinephrine. The Na^+/K^+ ATP'ase activity may be maintained for a sufficent period of time until protein synthesis recovers from the inhibition by CXM. Preliminary evidence from our laboratory suggests that CXM inhibition of leucine incorporation into protein is dissipating by 4 hr and is maximal only for 1 to 2 hr. Therefore labile memory traces need only be maintained for up to no more than 2 hr. The precise role of leucine uptake in memory formation, maintenance and inhibition is unclear.

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