The Influence of Chemical Agents on the Accumulation of Adenosine 3′,5′-Phosphate in Slices of Rabbit Cerebellum

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(Received December 18, 1967, and in revised form March 11, 1968)

SUMMARY

Incubation of slices of rabbit cerebellum with norepinephrine for a few minutes resulted in large increases in the tissue content of adenosine 3′,5′-phosphate. Exposure of slices to norepinephrine for more than 6 min led to a progressive disappearance of this nucleotide. Theophylline, a known inhibitor of cyclic-3′,5′-ribonucleotide phosphodiesterase, had relatively little effect by itself upon the slice content of adenosine 3′,5′-phosphate, but potentiated the effects of norepinephrine severalfold. In a survey of substances known to occur in the central nervous system, histamine, and to a lesser extent serotonin, were observed to produce effects similar to those of norepinephrine. The effects of histamine and norepinephrine were additive; phenoxybenzamine and diphenhydramine were observed to inhibit the effects of histamine at concentrations having relatively little influence on the action of norepinephrine while dichloroisoproterenol specifically prevented the effects of norepinephrine. Prolonged exposure of slices to norepinephrine desensitized slices to the readdition of norepinephrine, but not to histamine and vice versa.

INTRODUCTION

The formation and subsequent action of cyclic 3′,5′-AMP[2] have been implicated as essential steps in some of the metabolic and functional changes produced by a variety of hormones, many of which are of neural origin, e.g. catecholamines, ACTH, LH, and vasopressin (2–4). While comparatively little is known about hormonal influences on the metabolism of neural tissue, it has been observed that in mammals by far the greatest capacity to form cyclic 3′,5′-AMP resided in the central nervous system (5). Earlier studies using broken-cell preparations of brain adenyl cyclase showed that catecholamines produced an increased formation of the cyclic nucleotide (6). However, the effects were often small and usually amounted to no more than a 2-fold increase.

The present report represents the initial investigation of the possible role of cyclic 3′,5′-AMP in regulatory mechanisms in neural tissue, and begins with attempts to understand the factors governing tissue levels of the nucleotide.[3] In this tissue it will be necessary not only to identify which parameters of metabolic and functional alterations involve cyclic 3′,5′-AMP, but also to identify the hormonal and other factors producing the alterations. Since in earlier studies adenyl cyclase preparations from the cerebellum of various species usually possessed the largest response to catecholamines (6), the first series of experiments were performed on cerebellar tissue of a moderately large laboratory species, namely the rabbit.

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[2] Abbreviations used: cyclic 3′,5′-AMP, adenosine 3′,5′-phosphate; NE, L-norepinephrine (noradrenaline); INE, L-isoproterenol (isopropylnorepinephrine); DCI, dichloroisoproterenol [(3′,4′-dichlorophenyl)-2-isopropylaminoethanol].

[3] A preliminary report has appeared (1).

MATERIALS AND METHODS

Materials. L-(—)-Norepinephrine bitartrate hydrate was purchased from K and K Laboratories and Calbiochem; L-isoproterenol bitartrate dihydrate was obtained from Sterling-Winthrop Research Institute; histamine dihydrochloride, serotonin (5-hydroxytryptamine), creatinine sulfate, acetylcholine chloride, dopamine (3,4-dihydroxyphenylethylamine) hydrochloride, γ-aminobutyric acid, L-glutamic acid, anhydrous theophylline, and reserpine were purchased from Nutritional Biochemicals Corp.; cadaverine·2 HCl, putrescine·2 HCl, spermine·4 HCl, 1,4-methylhistamine (1-methyl-4-aminoethylimidazole)·2 HCl, 1-methylimidazole-4-acetic acid hydrochloride, and imidazole acetic acid hydrochloride were obtained from Calbiochem; spermidine phosphate·6 H_2O, and l-ergothionine hydrochloride·2 H_2O were purchased from Mann Research Laboratories; DCI was purchased from Aldrich Chemical Co. Phenoxybenzamine hydrochloride (Dibenzyline) and chlorpromazine hydrochloride (Thorazine) were generously supplied as pure solids by Smith, Kline and French, as was diphenhydramine hydrochloride (Benadryl) by Parke, Davis and Co. Normetanephrine and DL-octopamine were the gift of Dr. Marvin Armstrong, Fels Research Institute. Cyclic 3′,5′-AMP was purchased from Schwartz Bioresearch, Inc. Dowex-50H$^+$ (AG-50 × 8, 100–200 mesh) and Dowex-2 Cl (AG-2 × 8, 100–200 mesh) were obtained from Biorad Laboratories and were washed essentially as described previously (7), except that the number of cycles of washing with NaOH and HCl was doubled. Dipotassium glucose-1-P was supplied as a special grade by Nutritional Biochemicals Corporation after recrystallization by the procedure of Sutherland and Wosilait (8). Oyster glycogen, also obtained from Nutritional Biochemicals Corp., was reprecipitated before use (8). Disodium ATP and 5′-AMP were purchased from Pabst Laboratories. TES was purchased from Calbiochem.

Dephosphophosphorylase was prepared from dog liver by treatment of partially purified liver phosphorylase with phosphorylase phosphatase as described previously (9). Dephosphophosphorylase was also prepared directly from pig and beef liver by a modification of the procedure of Sutherland and Wosilait (8) used originally for the preparation of dog liver phosphorylase. The principal modifications[4] involved: (a) use of NaCl instead of NaF; (b) filtration of homogenates at pH 5.1 instead of 5.7; (c) omission of the heat-treatment step; (d) addition of glycogen (10 mg/ml) before removal of inactive protein at 40% saturation with $(NH_4)_2SO_4$. The procedure was terminated with the collection of the fourth alcohol fraction, modified as described previously (9). The crucial modification was filtration of homogenates at pH 5.1. Although decreasing the pH at this step from 5.7 reduced both the yield and final specific activity, it was found that this step removed material which interfered with the dephosphophosphorylase kinase reaction and the ability of cyclic 3′,5′-AMP to accelerate this reaction.

The liver supernatant fraction used in the assay of cyclic 3′,5′-AMP was prepared essentially as published previously (10) except that centrifugation was carried out at 15,000 *g* for 60 min and that incubation of the supernatant fraction before freezing was omitted. It should be pointed out that some preparations were not suitable for use in that the rate of conversion of dephosphophosphorylase to phosphorylase was high in the absence of cyclic 3′,5′-AMP and was increased relatively little by the addition of the cyclic nucleotide.

Incubation procedures. The animals used were male, New Zealand white rabbits, weighing between 2 and 3 kg. The rabbits were sacrificed by decapitation using a guillotine, and the cerebelli were removed and immersed in ice-cold incubation medium within 3 min after death. The tissue was transported to a cold room (3°) where it was dissected into blocks and

[4] The detailed procedure will be supplied upon request.

sliced using a McIlwain tissue chopper (Brinkmann Instrument Co.) with the blade adjustment set at 0.260 mm. The chopped tissue from 2 to 4 animals was pooled and transferred to a round-bottomed glass centrifuge bottle (100 ml capacity) containing about 30 ml of incubation medium. The contents of the bottle were swirled vigorously using a mechanical mixer and were poured into a basket made by stretching nylon mesh over a plastic ring (95 mm diameter) partially immersed in ice-cold incubation medium. Slices which continued to adhere together and any unchopped tissue could be readily detected and were removed. The dispersed slices were distributed to smaller baskets in portions estimated to be between 100 and 200 mg (approximately 15 to 25 slices). These baskets were made by stretching nylon mesh over a plastic ring with a diameter of about 35 mm. The mesh was held in place by a slightly larger ring with one serrated edge which served to elevate the nylon surface and allowed medium to move freely in and out of the basket. The baskets were kept partially immersed in ice-cold incubation medium until transferred one at a time to 100-ml beakers containing 30 ml of incubation medium and were shaken at 37° for 30 min with continuous gassing through glass tubes resting in the medium outside the baskets. The baskets containing the slices were then transferred to 20 ml of fresh medium in a second set of beakers and were shaken for an additional 12 min. This initial 42 min of incubation was considered a preincubation in order to reduce the level of cyclic 3′,5′-AMP to a stable minimum (11) and to restore the levels of phosphocreatine and monovalent ions to as near normal as is possible *in vitro* (12, 13). The incubations were considered to have commenced with the addition of 0.1 ml of hormone or suitable control solution and were terminated by removing and blotting the baskets followed by immersion into liquid N_2. The baskets were inverted in clean beakers containing liquid N_2 and the frozen slices were propelled into the liquid N_2 by scraping the nylon mesh with a plastic or metal spatula prechilled in liquid N_2. The slices were stored at −65° until being transferred to glass homogenizers as a slurry in liquid N_2 followed by homogenization and analysis for cyclic 3′,5′-AMP as described below. In experiments using tissue from four animals, the total elapsed time from the sacrifice of the first animal to the onset of the preincubation was between 1.5 and 2 hours.

The incubation medium consisted of 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2 mg of glucose per milliliter, and 0.02 mM Na_2EDTA. The solution was gassed during preparation and incubation with a mixture of 5% CO_2–95% O_2. Hormone solutions were prepared and diluted in 0.02 mM Na_2EDTA at pH 6.1. In experiments conducted in the presence of theophylline, this material was included in the medium beginning with the onset of the preincubation period unless specified otherwise (Table 1).

Estimation of tissue cyclic 3′,5′-AMP. The frozen slices were suspended in liquid N_2 and poured into a chilled glass Duall homogenizer, Size D (Kontes Glass Co.). The vessels were equilibrated at −20° and the tissue was homogenized rapidly after the addition of 15 ml of cold 0.025 N HCl containing 11 pmoles of ^{3}H-cyclic 3′,5′-AMP. Since it was noted that delays in achieving complete homogenization (i.e., longer than 10–20 sec) were often associated with cyclic 3′,5′-AMP values considerably lower than expected, the extraction procedure was later modified. The glass homogenizer was embedded in powdered dry ice and 2 ml of a mixture consisting of equal volumes of 0.38 N HCl in water and absolute ethanol was added, followed by the tissue as a slurry in liquid N_2. The vessel was removed from the dry ice and homogenization was accomplished as the mixture thawed (about −40°). ^{3}H-Cyclic 3′,5′-AMP and water were then added to bring the final volume to 18 ml, and the sample was rehomogenized. A small aliquot of homogenate was removed and analyzed for protein by procedures similar to those of Lowry *et al.* (14). The homoge-

nates were centrifuged at room temperature for 20 min at 8000 *g*, and the supernatant fluid (extract) was stored at −20°. The extracts could be stored at room temperature for at least 5 days without loss of cyclic 3′,5′-AMP.

The extracts were adjusted to pH 1.6 (glass electrode) and were filtered and diluted to 20 ml with 0.025 N HCl (pH 1.6). Each diluted extract was applied to a column packed with 6 ml of Dowex-50 (H^+) that had been equilibrated with 0.025 N HCl. The column diameter (i.d.) was 0.7 cm. After the sample, 25-ml and 40-ml portions of 0.025 N HCl were applied. At the start of application of the final (40 ml) portion of acid, the collection vessels were changed and the column was allowed to run dry. The final eluate fractions (40 ml) were filtered through a Millipore filter (pore size 0.45 μ), were frozen and taken to dryness *in vacuo*. The lyophilization flasks were extracted with 50 μl of 0.01 M Tris (pH 7.4) per milligram of protein in the original homogenate. If the appropriate volume was less than 0.5 ml, the flasks were extracted with 1.5 ml of H_2O and the resulting solution was transferred to 16 × 75 mm screw-top culture tubes and frozen and dried. The sample was reconstituted with no less than 100 μl of 0.01 M Tris (pH 7.4). The samples were assayed for radioactivity, as described by Butcher *et al.* (15), and for cyclic 3′,5′-AMP as outlined below. The fractionation procedure was designed to remove those tissue constituents known to interfere with the assay of cyclic 3′,5′-AMP, namely, hexose-phosphates and ADP (16). The average overall recovery of cyclic 3′,5′-AMP was about 50%. The total cyclic 3′,5′-AMP contained in each sample was calculated from the relationship expressed in Eq. 1. The initial

$$\text{pmoles cyclic 3',5'-AMP} = \frac{\text{initial specific activity}}{\text{final specific activity}} - 1 \times \text{pmoles } {}^3\text{H-cyclic 3',5'-AMP added} \quad (1)$$

specific activity of ^{3}H-cyclic AMP was determined periodically by subjecting aliquots of stock solutions to the exact purification procedure used for tissue samples.

Assay of Cyclic 3′,5′-AMP. The estimation of cyclic 3′,5′-AMP is based on the property of this compound to accelerate the conversion of dephosphophosphorylase to phosphorylase catalyzed by supernatant fractions of dog liver homogenates. The procedures used were scaled-down versions of those described previously (10, 17) and included the modifications described by Butcher *et al.* (15). The principal modifications[4] involved: (a) the use of 10 × 75 mm disposable glass culture tubes used only once after washing in very dilute detergent solutions; (b) increasing the concentrations of $MgSO_4$ and EDTA 3-fold and of ATP 2-fold during the first incubation (dephosphophosphorylase kinase reaction); (c) substitution of TES for Tris buffer; (d) decreasing the final volume of the first incubation from 300 μl to between 15 and 75 μl; (e) initiation of the second incubation (phosphorylase assay) by the addition of between 50 and 250 μl of a mixture of glucose-1-P, NaF, glycogen, and 5′-AMP adjusted to achieve the conditions originally prescribed (8); (f) the use of a Fisher Diluter to dilute 50 μl of the second incubation mixture with 3.2 ml of a mixture containing KI (0.6 mg), I_2 (0.3 mg), and HCl (0.013 meq). In order to avoid frequent appearance of spuriously high rates of phosphorylase formation it was found essential to avoid re-use of culture tubes or the use of new tubes soaked in detergent and to diminish the impact of CO_2 introduced during pipetting by increasing the buffering capacity of the reduced volume of the first incubation mixture.

On the basis of a preliminary assay, a dilution of each sample was prepared calculated to contain approximately 7 × 10^{-8} M cyclic 3′,5′-AMP. Two different-sized aliquots were assayed in duplicate; each aliquot was selected to achieve a final concentration of between 1.0 and 2.5 × 10^{-8} M in the first incubation. Appreciable and consistent deviation of the dose-response relationship of the sample solutions from that of standard solutions was taken as evidence for the presence of interfering substances and indicated the need for fur-

ther purification of the samples. Usually the values assigned to each of the four determinations in a single assay were within 10% of the mean value. Each value presented in the tables and figures represents a determination in a single aliquot of pooled slices and was calculated from the mean value observed in the assay.

Analysis of incubation media. In some experiments it was important to determine the accumulation of cyclic 3',5'-AMP in Krebs-Ringer bicarbonate solutions in which tissue samples had been incubated. An amount of concentrated HCl calculated to result in a final pH of about 1.5 was added to 20–60 ml of media. After the addition of 11 pmoles of ^{3}H-cyclic 3',5'-AMP and clarification by centrifugation at 8000 *g* for 20 min, the acidified samples were heated at 100° for 5 min, cooled and adjusted to pH 7.0–7.6 with KOH. If necessary, the samples were recentrifuged and applied to columns of Dowex-2 chloride 0.7 cm × 17 cm (6 ml of resin), previously equilibrated with glass-distilled water. Thereafter 40 ml of water and 80 ml of HCl at pH 2.25 were applied and the eluates were discarded. HCl, 20 ml, at pH 1.6 was applied, and the resultant eluates, which contained nearly 70% of the cyclic 3',5'-AMP applied, were collected and applied to columns of Dowex-50 as described above.

RESULTS

Effects of Norepinephrine and Theophylline

In preliminary experiments it was found that after 35–45 min of preincubation the level of cyclic 3',5'-AMP in cerebellar slices remained essentially constant during at least 60 min of subsequent incubation at 37°. Although such control values varied considerably from experiment to experiment (range 3.1–10.0 pmoles per milligram protein), within a given experiment the values fell within 15% of one another. In the presence of 1×10^{-4} M NE, the slice content of cyclic 3',5'-AMP rapidly increased, reaching a maximum within 6 min, and thereafter decreased at a rate resembling a first-order reaction. In the example shown in Fig. 1, the level increased by a factor of 6 after 6 min exposure to NE and fell to about twice the control value after 40 min. The inclusion of theophylline magnified the effect of NE about 3-fold, with little influence on the time course of

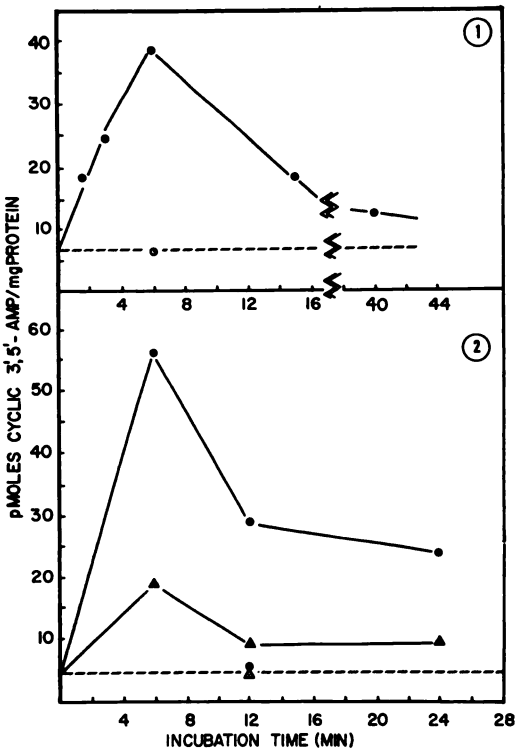

FIG. 1. (Top). *Time course of cyclic 3',5'-AMP content of cerebellar slices after addition of NE*

Slices had been incubated for 42 min prior to addition of sufficient NE to bring the concentration to 0.1 mM. The value observed in slices incubated a total of 48 min in the absence of NE was 6.4 pmoles per milligram of protein and is represented by the dashed line for reference.

FIG. 2. *Effect of theophylline on the accumulation of cyclic 3',5'-AMP in cerebellar slices incubated with NE*

Conditions as in Fig. 1. Incubation with 0.5 mM theophylline and 0.1 mM NE (●—●); incubation with NE alone (▲—▲). The values observed in the absence of NE after a total of 54 min of incubation in the presence (⊙) and absence (△) of 0.5 mM theophylline were 5.7 and 4.1 pmoles/mg protein, respectively, and are represented by the dashed line for reference.

the response (Fig. 2). Theophylline by itself had relatively small effect on the level of cyclic 3′,5′-AMP, producing values between 6% (Table 1) and 35% (Fig. 2) higher than controls.

TABLE 1
Effect of theophylline on accumulation of cyclic 3′,5′-AMP

Cerebellar slices were preincubated for 42 min at 37° with one change of medium. Sufficient medium containing 10 mM theophylline was added to achieve the indicated concentrations and the incubation was continued for 6 or 40 min.

Theophylline concentration (mM)	Incubation time (min)	Cyclic 3′,5′-AMP (pmoles/mg protein)
None	40	10.0
0.5	6	10.6
2.0	6	10.6
0.5	40	11.0

Qualitatively, certain of these results could have been predicted from the properties of broken-cell systems, namely, that NE would increase the accumulation of the cyclic nucleotide and that theophylline, an inhibitor of cyclic 3′,5′-nucleotide phosphodiesterase (18), would potentiate the effects of NE. On the other hand, the fact that theophylline by itself produced relatively small changes would appear to indicate that the rate of formation of cyclic 3′,5′-AMP in the absence of NE was quite low, a conclusion which is in sharp contrast to the behavior of brain adenyl cyclase preparations (6). Furthermore, both the magnitude and the transitory nature of the effects of NE were unexpected.

The time-course of the accumulation of cyclic 3′,5′-AMP was greatly influenced by the concentration of added NE. In the experiment depicted in Fig. 3, it can be seen that in the presence of 4×10^{-6} M NE the level of cyclic nucleotide had reached nearly 85% of the maximum level within 1½ min and after 15 min had fallen only 20% from that observed after 6 min of exposure to NE. By comparison, the addition of 1×10^{-4} M NE produced an increase of about 75% of maximum within 1½ min and the level fell more than 35% in the interval between 6 and 15 min. This experiment indicated that 6 min of exposure to NE would produce the maximal effect of a given concentration and that under these conditions, 4×10^{-6} M NE produced approximately 50% of the maximal effect. The latter conclusion was supported by other experiments. In the majority of the remaining experiments to be presented, 5×10^{-4} M theophylline was included in the incubation media in order to magnify the accumulation of cyclic 3′,5′-AMP resulting from factors which might increase the rate of synthesis and to diminish the impact of factors which might inhibit the breakdown of this substance via cyclic 3′,5′-nucleotide phosphodiesterase.

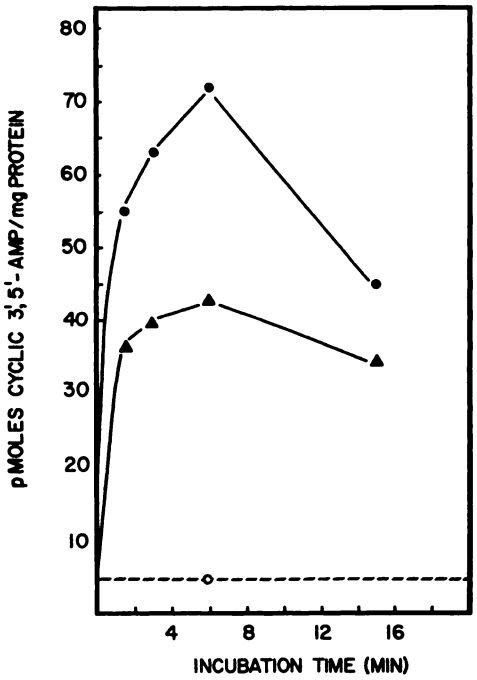

FIG. 3. *Influence of NE concentration on accumulation of cyclic 3′,5′-AMP in cerebellar slices*

Slices had been incubated for 42 min prior to addition of sufficient NE to achieve a concentration of 100 μM (●—●) or 4 μM (▲—▲). The value observed after a total of 48 min of incubation in the absence of NE was 4.7 pmoles/mg protein and is represented by the dashed line for reference. All incubation media contained 0.5 mM theophylline.

Effects of Histamine and Isoproterenol

In a survey of a number of agents considered possible candidates for mediators of chemical transmission in the central nervous system, histamine was observed to produce large increases in the accumulation of cyclic 3',5'-AMP. In Fig. 4 are summarized the data of an experiment comparing the effects of histamine, NE and INE, as well as of NE and histamine combined, as a function of time. It can be seen that the level of cyclic 3',5'-AMP increased about 9-fold within 6 min after the addition of 1×10^{-4} M histamine and decreased thereafter in a fashion similar to that in slices exposed to NE. Furthermore, the changes produced by the addition of histamine and NE together were at least as great as that calculated from the sum of the effects produced by the individual agents except for the values observed after 40 min of incubation. In a similar experiment after 30 min of incubation the calculated and observed values were almost exactly the same. Using 6 min of exposure, 1×10^{-5} M histamine usually produced nearly half-maximal effects (Table 2).

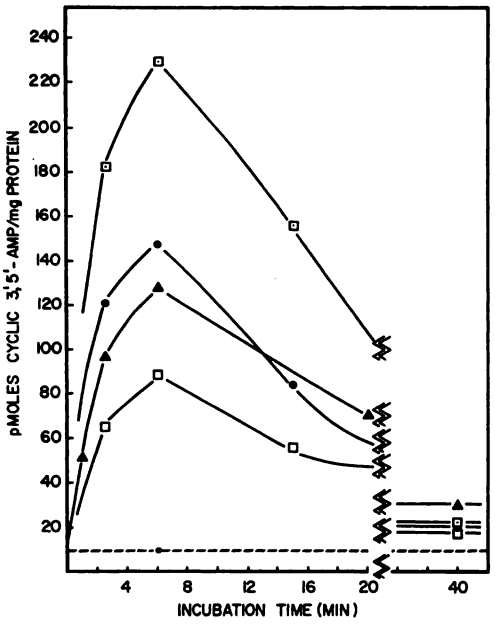

FIG. 4. *Time course of cyclic 3',5'-AMP content of cerebellar slices following addition of NE, histamine, or INE*

Slices had been incubated for 42 min prior to addition of sufficient NE (●—●) or INE (▲—▲) to achieve a final concentration of 50 μM or histamine (□—□) to reach 100 μM. The upper curve (⊡—⊡) represents the values observed after the simultaneous addition of NE (50 μM) and histamine (100 μM). The value observed after incubation in the absence of the amines for a total of 48 min was 9.3 pmoles per milligram of protein and is represented by the dashed line for reference. All incubation media contained 0.5 mM theophylline.

TABLE 2

The relation of histamine concentration and the accumulation of cyclic 3',5'-AMP

Cerebellar slices were preincubated 42 min followed by 6 min exposure to histamine at the indicated concentrations. All incubation media contain 0.5 mM theophylline.

Histamine concentration (mM)	Cyclic 3',5'-AMP (pmoles/mg protein)
0.0	3.9, 4.1
0.001	5.4
0.01	16.2
0.10	34
1.0	34

TABLE 3

Accumulation of cyclic 3',5'-AMP in incubation media

After removal of slices (data in Fig. 4), the incubation media were acidified, supplemented with ^{3}H-cyclic 3',5'-AMP and processed as described in the text. The results are expressed as amount of cyclic 3',5'-AMP per milligram of protein contained in the slices which had been incubated in each medium.

Hormone	Incubation time (min)	Medium cyclic 3',5'-AMP (pmoles/mg protein)
None	6	8.9
NE	6	11.6
Histamine	6	11.0
NE + histamine	6	10.3
NE	15	15.5
Histamine	15	11.5
NE + histamine	15	24
NE	40	27
Histamine	40	23
NE + histamine	40	35

TABLE 4

Effect of exposing cerebellar slices to multiple hormone additions

After 42 min preincubation in the presence of 0.5 mM theophylline, hormone solutions were added after 0 min and/or 34 min of incubation in an amount calculated to increase the hormone concentration by 0.1 mM, and all incubations were terminated after a total of 40 min. Samples number 9 and 10 were incubated for 6 min in the medium remaining after removal of samples number 3 and 4, respectively. Samples number 11 and 12 were incubated with hormone for 5 min, were rinsed in 50 ml of fresh medium for 1 min at room temperature and were incubated in two 25-ml portions of fresh medium for 18 and 10 min successively before readdition of hormone.

Sample number	First addition, at 0 min	Second addition, at 34 min	Cyclic 3′,5′-AMP (pmoles/mg protein)
1	None	NE	105[a]
2	None	Histamine	47
3	NE	None	28
4	Histamine	None	19
5	NE	NE	23
6	Histamine	Histamine	20
7	NE	Histamine	94
8	Histamine	NE	98
9	None	(To medium No. 3)	91
10	None	(To medium No. 4)	41
11	NE (for 5 min only)	NE	49
12	Histamine (for 5 min only)	Histamine	31

[a] Slices incubated without hormone for 10, 20, and 40 min contained 6.3, 6.9, and 6.5 pmoles cyclic 3′,5′-AMP per milligram of protein, respectively.

The effects of INE were more complex. The maximal levels of cyclic 3′,5′-AMP in slices exposed to INE for short periods (2.5–6 min) were between 13% and 63% lower than in those exposed to NE (4 experiments). However, after 40 min exposure to INE the levels were between 23% and 45% greater than after similar treatment with NE (4 experiments). Although in other experiments it appeared that INE produced its maximal effect at a lower concentration than that required for a maximal effect using NE, it was not possible to make a reasonable estimate of the relative potency of the two agents. In earlier studies using broken-cell preparations from sheep cerebellum, INE was found to be at least 10 times as potent as NE in stimulating the formation of cyclic 3′,5′-AMP (6). Observations on the effectiveness of epinephrine with cerebellar slices have not been made as yet.

In an effort to understand the mechanisms underlying the influence of time upon the accumulation of cyclic 3′,5′-AMP in slices, it was necessary to exclude the possibility that translocation into the incubation media could be involved to an appreciable degree in the disappearance of the nucleotide after the initial 6 min of exposure to a stimulating agent. In Table 3 are summarized the analyses of some of the incubation media from the experiment of Fig. 4. It can be calculated that between 6 and 40 min of incubation the amount of cyclic 3′,5′-AMP appearing in the medium was about 12% of the amount disappearing from slices exposed to NE or to NE and histamine together, and less than 19% in slices exposed to histamine alone. The experiment described in Table 4 was performed to eliminate some other possible explanations of this phenomenon. First, disappearance of the agent itself can be discounted since readdition of the same agent did not increase the level of cyclic 3′,5′-AMP reached after 40 min of incubation. Second, accumulation of toxic or inhibitory

materials cannot be primarily responsible since large accumulations of the nucleotide were observed in slices incubated for 6 min in media in which other slices had been incubated with NE or histamine for 40 min. This is also additional evidence that sufficient stimulating agent was present to sustain the response. Third, general deterioration of the slices could not be an important factor since incubation of slices for 34 min in the absence or in the presence of one agent did not prevent the addition of the other agent from producing a large change during the final 6 min. Furthermore, interpretations of this phenomenon will have to take into account the observation that 5 min of exposure to an agent followed by rinsing and incubation in the absence of the agent resulted in a severely reduced response upon readdition of the agent.

Selective Inhibition of Effects of NE and Histamine

From the data presented in Fig. 4 and Table 4 it would appear that NE and histamine exert their effects independently of each other. Another observation, in which incubation of slices with 5×10^{-5} M INE for 34 min prevented completely any increase in the level of cyclic 3′,5′-AMP upon the subsequent addition of 5×10^{-5} M NE, is consistent with the interpretation that INE exerts its effects by interaction with a receptor for NE. If this idea is valid, then it should be possible to find blocking agents which will inhibit the effects of NE and not histamine and vice versa. In Table 5 are summarized a series of experiments on the effectiveness of several pharmacologically active materials in preventing responses to approximately half-maximal doses of either NE or histamine. It can be seen that the antihistamine, diphenhydramine (Benedryl), can nearly obliterate the effect of histamine under conditions in which the response to NE is reduced by only 20–30%. Using the β-adrenergic blocking agent, DCI, just the reverse was true. Phenoxybenzamine (Dibenzyline), usually considered an α-adrenergic blocking agent but known to possess atropinic, antihistaminic, and antiserotinin properties as well (19), appeared to antagonize the effects of histamine relatively specifically. Chlorpromazine, on the other hand, was observed to inhibit the effects of both agents, although higher concentrations appeared to be required to achieve the same degree of inhibition of the effects of NE than of histamine. While the conclusion that histamine and NE act independently may be correct to the first approximation, there are indications that the situation is more complex. The cross-reactions of the "specific" inhibitors either may be due to a degree of nonspecificity of the inhibitors or may indicate that a component of the effect of one agent involves in some way the sites which interact predominantly with the other agent. It should be pointed out that experiments of the type described in Table 5 do not distinguish effects due to inhibition of the rate of penetration of the active agent to the immediate vicinity of the receptor sites from those due to direct interaction of the inhibitor with the receptor sites.

Effects of Other Agents

In preliminary experiments, a number of substances found in central nervous system tissue were tested for their effect upon the level of cyclic 3′,5′-AMP in cerebellar slices in the presence of theophylline and were found either to have no effect or to produce increases of less than 30%. These included dopamine, acetylcholine (and carbamylcholine), octopamine, histidine, imidazole acetate, 1-methylhistamine, 1-methylimidazole acetate, cadavarine, putrescine, spermine, spermidine, normetanephrine, ergothionine, γ-aminobutyrate, and L-glutamate. The latter two substannes were tested at 4×10^{-4} to 4×10^{-3} M, while the remainder were tested at 1 to 5×10^{-4} M concentrations.

By contrast, serotonin (5-hydroxytryptamine) produced significant elevations in cyclic 3′,5′-AMP. In five experiments in which slices were exposed to between 1×10^{-5} M and 1×10^{-3} M serotonin for 6 min, cyclic 3′,5′-AMP levels were increased by $276 \pm 30\%$ ($p < 0.01$; control values

TABLE 5

Inhibition of effects of NE and histamine

Cerebellar slices were preincubated for 30 min and were transferred to fresh medium containing the inhibitor at various concentrations. After incubation for 12 min, enough NE or histamine was added to achieve a final concentration of 5 μM and 10 μM, respectively and the incubation was continued for 6 min. All incubations were carried out in the presence of 0.5 mM theophylline.

Agent	Inhibitor	Concentration (μM)	Cyclic 3′,5′-AMP (pmoles/mg protein)	Inhibition (%)
Experiment I				
None	None	—	8.0	—
Histamine	None	—	40	—
Histamine	DCI	1	34	20
Histamine	DCI	10	30	33
Histamine	Chlorpromazine	5	22	57
Histamine	Chlorpromazine	50	13.1	84
Histamine	Diphenhydramine	5	16.3	74
Histamine	Diphenhydramine	50	9.2	96
Experiment II				
None	None	—	8.2	—
NE	None	—	99	—
NE	Diphenhydramine	5	81	20
NE	Diphenhydramine	50	73	29
NE	Phenoxybenzamine	5	87	13
NE	Phenoxybenzamine	50	104	0
NE	Phenoxybenzamine	500	77	24
Experiment III				
None	None	—	3.1	—
NE	None	—	76	—
NE	DCI	0.5	13.8	85
NE	DCI	5	3.9	99
Experiment IV				
None	None	—	7.7	—
Histamine	None	—	25	—
Histamine	Phenoxybenzamine	5	23	11
Histamine	Phenoxybenzamine	50	8.2	97
Histamine	Chlorpromazine	50	10.3	85
NE	Chlorpromazine	5	31	21
NE	Chlorpromazine	50	24	47
NE	None	—	38	—

ranged from 3.4 to 4.0 pmoles/mg protein). Since exposure to the agent for 40 min yielded values less than 25% larger than controls, it is likely that the changes produced by serotonin follow a time course similar to that for NE and histamine.

In order to acquire evidence bearing on the possibility that serotonin exerted its effects primarily via the release of NE, an experiment using tissue from reserpinized animals was performed (Table 6). Cyclic 3′,5′-AMP was increased by 234% in slices exposed for 6 min to serotonin; this result falls within one standard deviation of the average result for nonreserpinized tissue. The effects of NE and histamine also appear not to be appreciably diminished by reserpine treatment. This experiment was not intended to detect a possible increase in the effectiveness of NE or histamine in reserpinized tissue; any test of this possibility should include evaluation of changes

in dose-response relationships as well as changes in maximal responses.

Since Murad *et al.* (20) had observed that cholinergic agents reduced the formation of cyclic 3′,5′-AMP by broken-cell preparations of cardiac muscle, an attempt to make similar observations with cerebellar slices was made. However, exposure of slices to 1×10^{-4} to 1×10^{-3} M acetylcholine (and 5×10^{-4} M eserine) for 12–32 min did not appreciably alter the effects of adding either NE or histamine on the accumulation of cyclic 3′,5′-AMP.

TABLE 6

Accumulation of cyclic 3′,5′-AMP in cerebellar slices obtained from rabbits treated with reserpine

Three rabbits were injected subcutaneously on three successive days with 0.75 mg/kg of reserpine (dissolved in 20% ascorbic acid). The animals were sacrificed 18 hr after the last injection. After 42 min of preincubation in the presence of 0.5 mM theophylline, hormone was added to bring the final concentration to 0.1 mM.

Hormone	Incubation time (min)	Cyclic 3′,5′-AMP (pmoles/mg protein)
None	6	3.9
Serotonin	2	10.4
Serotonin	6	13.0
NE	2	78
NE	6	121
Histamine	6	76

DISCUSSION

The general finding that the level of cyclic 3′,5′-AMP in cerebellar tissue is subject to fairly rapid and marked fluctuations may be sufficient excuse to continue investigations based on the hypothesis that the nucleotide is an important regulatory substance in the central nervous system. More extensive interpretations based on some of the specific findings may be quite premature. For example, much more evidence must be obtained before it is possible to relate the effects of NE, histamine, and serotonin described here to events taking place *in vivo*. At present, the evidence supporting the notion that any one of the three amines functions in neurohumoral transmission processes is meager. Mammalian cerebellum has been found to contain relatively small amounts of the three amines [for references see McLennan (21)]. However, Iversen and Glowinski (22) concluded that the turnover of NE in rat cerebellum was nearly twice that in the hypothalamus even though the steady-state level was only about one-tenth as great. In addition, Fuxe (23), using histochemical fluorescence methods, has observed nerve endings containing NE scattered throughout rat cerebellum. In attempting to connect these observations with stimulation of cyclic 3′,5′-AMP accumulation by NE, it will be important to determine whether this response takes place to a significant degree in neuronal cell bodies and dendritic processes.

Another important and related question is whether the effects of the three amines were predominantly direct or indirect. It is most unlikely that the effects of histamine involved the release of NE to any appreciable degree. The observation that serotonin was effective in slices prepared from animals treated with reserpine would diminish but not remove the possibility of involvement of endogenous NE. It is also possible that the effects of all three amines were achieved primarily as the result of the release and action of unknown endogenous substances. One explanation of the evanescent changes in cyclic 3′,5′-AMP accumulation could be the depletion of such essential substances. However, in a preliminary experiment, exposure of cerebellar slices to 100 mM KCl for 10 min in the presence of theophylline increased cyclic 3′,5′-AMP levels by no more than 30%. Such treatment, involving depolarization of neuronal elements, might be expected to release potentially active substances.

The decline of cyclic 3′,5′-AMP in cerebellar slices after exposure for about 6 min to NE or histamine obviously must result from either a reduction in the rate of synthesis or an increase in the rate of metabolism of the compound. While there is little direct evidence which would distinguish between these possibilities, the latter explanation would seem to require the

more complicated interpretation of existing data. Since theophylline had little effect upon the time course of responses to NE and histamine, it would be necessary to propose that the metabolic pathways involved would not be sensitive to the methylxanthines and thus would presumably exclude changes in rate of the only known pathway, namely that involving the cyclic ribonucleotide phosphodiesterase. It would also be necessary to compartmentalize units responding to each agent, perhaps placing them in different cells, in order to explain the relative lack of effect of prolonged exposure to one agent upon the response to the other. Finally, it would be necessary to propose some sort of a time-dependent mechanism which linked an effect of the hormonal agent (such as the accumulation of cyclic 3′,5′-AMP) to a change in the kinetic parameters of metabolism of this nucleotide. The simpler proposition that the waxing and waning levels of cyclic 3′,5′-AMP induced by the hormones reflect primarily waning and waxing rates of metabolism in the presence of constant synthetic flux would also appear to be unlikely. The relatively large and small effects of theophylline in the presence and absence of the hormones, respectively, would again require that a theophylline-insensitive pathway for the metabolism of the nucleotide exist. Future studies will include attempts to estimate the turnover of cyclic 3′,5′-AMP in order to provide more direct evidence on these questions.

ACKNOWLEDGMENTS

This work was supported in part by Grants AM-06141, NB-05716, and GM-661 from the U.S. Public Health Service.

The authors also wish to acknowledge the skillful technical assistance of Mrs. Arleen Maxwell Haley and Mr. George Thorne in carrying out this work.

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