Cellular Site and State of Combination of the Adenosine 3':5'-Cyclic Monophosphate Persisting after Excitation of Cerebral Tissues

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1. The subcellular distribution of cyclic AMP in guinea-pig cerebral-cortical tissue was determined before and after electrical stimulation, and after a period of continued incubation after electrical stimulation, with and without the presence of histamine. 2. Electrical stimulation and histamine increased the cyclic AMP content of all fractions, the greatest increase occurring in the supernatant fraction. 3. Continued incubation after cessation of electrical stimulation diminished the cyclic AMP of the total homogenate and supernatant fractions, but increased that of the synaptosomal fraction. 4. Further fractionation of the synaptosomal fractions from stimulated tissues suggested that most of their soluble cyclic AMP was in a higher-molecular-weight form than was that of the tissue supernatant. 5. It is suggested that protein binding and cytoplasmic transport of cyclic AMP are involved in the changes observed.

Neural systems are among the richest mammalian sources of cyclic AMP and of enzymes that synthesize and degrade it (Robison *et al.*, 1970). The present investigations were prompted by noting that the nucleotide has been implicated in neural processes which differ greatly in nature and time course. These include alterations in postsynaptic potentials, which in some instances may last for a few milliseconds, and in others for 1 h (Libet *et al.*, 1975; Kuroda *et al.*, 1976; Greengard, 1976), and changes in glycogen content during 90 min (Wilkening & Makman, 1976). Determination of cyclic AMP in association with these and other processes (Barberis *et al.*, 1976) as observed in cerebral systems has usually been performed after acid extraction of entire tissues, and thus did not give information on localized or combined forms of the nucleotide that might affect the time course of its action. Such information has now been sought by performing subcellular fractionation, gel filtration or other procedures before determining the cyclic AMP of neocortical tissues; the tissues were maintained *in vitro* and some were electrically stimulated or exposed to histamine to increase their content of cyclic AMP (Kakiuchi *et al.*, 1969; Schultz & Daly, 1973).

In performing subcellular fractionation, the conditions first adopted were those under which incubated tissues have been found to yield normal subcellular fractions (Kuroda & McIlwain, 1973; Barberis & McIlwain, 1976); however, these led to loss of cyclic AMP, and a preliminary investigation was needed to find conditions for its preservation without greatly altering the fractionation method. The conditions adopted used inhibitors of cyclic

nucleotide phosphodiesterases, and the distribution of materials in the presence of these inhibitors is reported first.

Experimental

Tissue preparation and separation

Guinea-pig cerebral-cortex slices were prepared, superfused and electrically stimulated as described by Pull & McIlwain (1973). Subcellular fractionation was performed (see also Barberis & McIlwain, 1976) with the use of an SW50 rotor in a Spinco model L ultracentrifuge. A 10% (w/v) homogenate of the slices in 0.32 M-sucrose containing 0.5 mM-EDTA and phosphodiesterase inhibitors as described in the Results section was centrifuged at 1000g for 10 min to deposit the nuclear fraction, and the supernatant from this was further centrifuged at 10000g for 20 min to pellet the crude mitochondrial fraction. This was resuspended in 1 ml of 0.32 M-sucrose and layered on a gradient of 2 ml of 0.8 M-, 2 ml of 1.2 M- and 0.3 ml of 1.4 M-sucrose, which was centrifuged at 105000g for 60 min. The synaptosomal fraction, containing 2-4 mg of protein/ml, was collected by using a bent-tip Pasteur pipette with suction. In some experiments it was sonicated for 30 s at 1.4 A and centrifuged at 130000g for 60 min in a type-65 head to yield cytoplasmic and vesicular components, as described by Maynert *et al.* (1964).

Gel filtration of supernatants after sonication was performed on columns (11.0 cm × 0.8 cm) containing 5 ml of Sephadex G-25 equilibrated with 50 mM-Tris/HCl/8 mM-theophylline/6 mM-mercaptoethanol,

pH 7.4. Samples were applied in a volume of 0.5 ml, and ten 0.5 ml fractions collected after elution of the void volume (2.0 ml; determined by calibration with Blue Dextran).

Analyses

Protein was determined by the method of Miller (1959), and radioactivity determinations were as described by Newman & McIlwain (1977). Cyclic AMP in trichloroacetic acid extracts of the tissue fractions was determined by the method of Brown *et al.* (1971). The presence of sucrose (0.32–1.4 M) or 3-isobutyl-1-methylxanthine (5 mM) had no effect on binding of cyclic [^3H]AMP in the assay.

Exposure of extracts to cyclic nucleotide phosphodiesterase

The soluble cytoplasmic (S_1) and the synaptosomal fractions were subjected to digestion by the phosphodiesterase preparation described below, at a final concentration of 0.1 mg/ml, and for 30 min at 37°C, after removal of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine by five extractions with an equal volume of water-saturated diethyl ether. Removal of the xanthine was indicated by a successive decrease in A_{293} of the extract, when read against a solution containing amounts of sucrose and xanthine equivalent to those present in the tissue supernatants after successive ether extraction.

Reagents

Cyclic [$8\text{-}^3\text{H}$]AMP (27 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Histamine and theophylline were from BDH Chemicals, Poole, Dorset, U.K.; papaverine hydrochloride was from Hopkin and Williams, Chadwell Heath, Essex, U.K.; 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3':5'-Cyclic AMP 5'-nucleotidohydrolase

(EC 3.1.4.17) from ox heart and unlabelled nucleotides were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Sephadex G-25 was from Pharmacia (G.B.), London W.5, U.K.

Results

Preservation of cyclic AMP in tissue dispersions: centrifugal separation

The cyclic AMP content of homogenates of cortical tissue in 0.32 M-sucrose had decreased to 4% of the original values after the time necessary to complete a subcellular fractionation under ordinary conditions (Table 1). Preservation of cyclic AMP was improved by inclusion of 8 mM-theophylline or 5 mM-papaverine in the homogenization medium, but attempted subcellular fractionation in the presence of the latter compound led to a very different distribution of the tissue's protein compared with that of tissue fractionated in sucrose alone. 3-Isobutyl-1-methylxanthine (5 mM) was considerably more effective than either of the other compounds tested in preserving the cyclic AMP of the total homogenate (Table 1), and its inclusion did not alter the protein distribution of the primary fractions. Thus in sucrose the nuclear fraction contained $47.1 \pm 2.0\%$ of the protein recovered, the crude mitochondrial $28.9 \pm 1.8\%$, and the supernatant $23.8 \pm 0.2\%$. In fractions obtained by centrifugation in sucrose and methylisobutylxanthine the nuclear fraction contained $47.0 \pm 0.9\%$, the crude mitochondrial $28.4 \pm 1.7\%$, and the supernatant $24.4 \pm 0.8\%$ (means $\pm$ S.E.M. for three determinations in each case). Recovery of protein was 95–98% of that in the original dispersion and was also unaffected by the xanthine, which was therefore included in subsequent experiments. Addition of a fixed amount of cyclic AMP to the tissue homogenate before fractionation resulted in almost complete recovery of the added material in the supernatant fraction (Table 2), with virtually no uptake by any of the particulate fractions including the synaptosomal fraction.

Table 1. Cyclic AMP of cerebral dispersions

Tissues were preincubated and then superfused at 37°C for 16–17 min with media containing 0.1 mM-histamine, and stimulated for the last 4–6 min of superfusion. They were quickly dispersed at 0°C in 10 vol. of 0.32 M-sucrose containing 0.5 mM-EDTA with the other additions specified, and the homogenates were sampled for analysis by adding 200 μl to 0.3 ml of sucrose plus 0.5 ml of 10% (w/v) trichloroacetic acid at the times specified. The initial cyclic AMP was 280–400 pmol/mg of protein, and each value below gives the mean $\pm$ S.E.M. for three experiments.

Addition to dispersion fluid (mM)	Time at 0°C (min)	Cyclic AMP remaining (%)		
		20	60	240
None	...	—	—	3.8
Theophylline (8)		95.5 ± 1.4	81.9 ± 3.6	22.2 ± 2.3
Papaverine (5)		88.1 ± 12.8	72.2 ± 3.6	58.8 ± 1.8
Isobutylmethylxanthine (5)		91.8 ± 4.5	92.7 ± 3.9	87.6 ± 2.2

Table 2. *Recovery after subcellular fractionation of cyclic AMP added to tissue dispersions*

Tissues (4, weighing 300–420mg collectively in each experiment) were incubated for 30min and superfused for 20min before being homogenized in 0.32M-sucrose/5mM-isobutylmethylxanthine. Each homogenate was divided into equal parts (a) and (b), and 1.5 nmol of cyclic AMP added to (b); subcellular fractionation was carried out as described in the Experimental section. Differing tissue weights are responsible for the differing values (a). The mean value in the final column is the mean for the three experiments $\pm$ S.E.M.

Fraction	Cyclic AMP (nmol)						Mean value (b)–(a)
	Expt. 1		Expt. 2		Expt. 3		
	(a)	(b)–(a)	(a)	(b)–(a)	(a)	(b)–(a)	
Homogenate	1.54	1.11	1.91	1.57	2.13	1.63	1.44 ± 0.22
Nuclear	0.41	–0.01	0.67	0.29	0.85	–0.16	0.04 ± 0.13
Crude mitochondrial	0.56	0.06	0.37	–0.02	0.36	0.03	0.02 ± 0.01
Supernatant	0.33	1.31	0.33	1.76	0.14	1.11	1.39 ± 0.24
Synaptosomal	0.15	–0.04	0.04	0.03	0.05	0.01	0.01 ± 0.02

Table 3. *Cyclic AMP and protein in dispersions of stimulated and unstimulated tissues*

Tissues received preliminary incubation and superfusion as stated in the legend to Table 1, followed by the additional treatments specified below. Results of subcellular fractionation gave mean values $\pm$ S.E.M. for the numbers of experiments in parentheses. Significantly different from synaptosomal value for unstimulated tissues: * $P < 0.025$, ** $P < 0.01$ (Student's *t* test).

Fraction	Cyclic AMP of tissues with:					
	No additional treatment (7)		Stimulation (10min) (4)		Stimulation (10min) and further incubation (6min) (5)	
	(pmol/mg of protein)	(% of homogenate)	(pmol/mg of protein)	(% of homogenate)	(pmol/mg of protein)	(% of homogenate)
Homogenate	54 $\pm$ 5	—	165 $\pm$ 32	—	139 $\pm$ 31	—
Nuclear	52 $\pm$ 5	51.2 $\pm$ 7.0	68 $\pm$ 11	26.0 $\pm$ 8.7	71 $\pm$ 12	36.6 $\pm$ 10.1
Crude mitochondrial	86 $\pm$ 11	25.2 $\pm$ 5.5	181 $\pm$ 65	22.1 $\pm$ 10.8	86 $\pm$ 19	11.8 $\pm$ 1.6
Supernatant	78 $\pm$ 18	24.5 $\pm$ 3.9	325 $\pm$ 45	35.8 $\pm$ 4.6	226 $\pm$ 35	32.9 $\pm$ 4.0
Synaptosomal	53 $\pm$ 13	6.2 $\pm$ 1.6	126 $\pm$ 34*	3.7 $\pm$ 1.1	167 $\pm$ 48**	5.2 $\pm$ 1.6

Subcellular distribution after stimulation, and on continued incubation

Electrical stimulation of tissues increased the cyclic AMP per unit protein, in homogenates prepared from the tissues in the presence of the methylisobutylxanthine (Table 3). When incubation and superfusion were continued after stimulation had been discontinued, the cyclic AMP of derived homogenates diminished, but was still 84% of its immediate post-stimulation value 6min later.

Subcellular fractionation of tissues sampled before and after this stimulation showed that the cyclic AMP in their nuclear fractions remained relatively constant, and the greater part of the increase in the nucleotide was associated with components of the crude mitochondrial and supernatant fractions. When the crude mitochondrial preparation was further fractionated and the synaptosomal fraction separated, the increase in its cyclic AMP content observed immediately after stimulation was much smaller than

the tissue average. However, in tissues taken 6min after stimulation, this relationship was reversed: the synaptosomal fraction now had markedly more cyclic AMP per unit of protein than the tissue average. Further incubation of tissues for 13min after electrical stimulation led to a fall in cyclic AMP content, to 110pmol/mg of protein for the whole homogenate and 57pmol/mg of protein for the synaptosomal fraction.

Incubation of tissues in the presence of 0.1mM-histamine caused 30% increase in the cyclic AMP per unit of protein of resultant homogenates, without appreciable alteration of the distribution on primary fractionation. Further separation showed, however, a preferential increase in cyclic AMP in the synaptosomal fraction (Table 4). Electrical stimulation in the presence of histamine now gave a large, 6.5-fold, increase in cyclic AMP above basal values; this diminished gradually in most fractions during the 13min after stimulation in which observations were made and

Table 4. Cyclic AMP and protein in dispersions of tissues incubated with 0.1 mM-histamine

Tissues were incubated for 30 min in glucose/bicarbonate saline (Newman & McIlwain, 1977) and superfused for 20 min in medium containing 0.1 mM-histamine followed by the additional treatments specified below in the continued presence of histamine. Results of subcellular fractionation are means $\pm$ S.E.M. for four observations.

Fraction	Cyclic AMP of tissues with:							
	No additional treatment		Stimulation (4 min)		Stimulation (4 min) and further incubation (6 min)		Stimulation (4 min) and further incubation (13 min)	
	(pmol/mg of protein)	(% of homogenate)	(pmol/mg of protein)	(% of homogenate)	(pmol/mg of protein)	(% of homogenate)	(pmol/mg of protein)	(% of homogenate)
Homogenate	70 $\pm$ 18	—	348 $\pm$ 79	—	213 $\pm$ 25	—	218 $\pm$ 29	—
Nuclear	80 $\pm$ 3	30.3 $\pm$ 6.2	158 $\pm$ 49	21.0 $\pm$ 2.4	81 $\pm$ 13	16.8 $\pm$ 3.6	98 $\pm$ 15	21.7 $\pm$ 3.1
Crude mitochondrial	83 $\pm$ 9	25.9 $\pm$ 9.4	219 $\pm$ 74	15.7 $\pm$ 3.3	178 $\pm$ 7	10.0 $\pm$ 8.5	204 $\pm$ 70	16.2 $\pm$ 2.3
Supernatant	109 $\pm$ 17	33.8 $\pm$ 9.5	571 $\pm$ 122	30.2 $\pm$ 1.7	529 $\pm$ 65	57.9 $\pm$ 1.6	309 $\pm$ 47	28.2 $\pm$ 2.9
Synaptosomal	136 $\pm$ 3	8.6 $\pm$ 3.5	214 $\pm$ 33	3.8 $\pm$ 0.6	157 $\pm$ 8	3.5 $\pm$ 0.9	151 $\pm$ 10	7.5 $\pm$ 1.5

without unusual relationships in the synaptosomal fraction.

Protein distribution was measured in the experiments of Tables 3 and 4 and found not to be altered by the stimulation and histamine used. The recovery of cyclic AMP during primary fractionation in these experiments was $85.5 \pm 4.0\%$ (mean $\pm$ S.E.M. for 32 observations).

Properties of cyclic AMP in supernatant and synaptosomal fractions

The synaptosomal fraction of tissues that had been electrically stimulated was sonicated and centrifuged by the procedure of Maynert *et al.* (1964), which is designed to obtain cytoplasmic and vesicular components (see the Experimental section); the soluble component contained $79.4 \pm 7.8\%$ of the total

Fig. 1. Sephadex-elution pattern of cyclic AMP from two subcellular fractions

The synaptosomal and part of the supernatant fractions derived from subcellular fractionation of approx. 400 mg of stimulated tissues corresponding to those of Table 3 were sonicated and further centrifuged as described in the Experimental section. Of each supernatant, 0.5 ml was applied to columns (0.8 cm $\times$ 11.0 cm) containing 5 ml of Sephadex G-25, followed by 50 mM-Tris/8 mM-theophylline/6 mM-mercaptoethanol, pH 7.4, and 0.5 ml fractions were collected and assayed for cyclic AMP and protein. ○, Synaptosomal supernatant; ▲, tissue supernatant.

Fig. 2. Susceptibility of cyclic AMP to phosphodiesterase hydrolysis

Supernatant fractions from incubated and stimulated tissues, and from their derived synaptosomes, were extracted with ether to remove 3-isobutyl-1-methylxanthine and incubated at 37°C in the presence of 0.1 mM-MgCl₂, 0.1 mM-CaCl₂ and 0.1 mg of ox heart phosphodiesterase/ml. Samples were withdrawn at 15 and 30 min and added to equal volumes of 10% (w/v) trichloroacetic acid for determination of cyclic AMP. ■, Standard solution of cyclic AMP in 0.32M-sucrose; ▲, tissue supernatant; ○, synaptosomal supernatant.

cyclic AMP recovered and $52.0 \pm 6.4\%$ of the total protein recovered, whereas the pellet or vesicle component contained $20.5 \pm 7.8\%$ of cyclic AMP and $48.0 \pm 6.4\%$ of protein (mean $\pm$ S.E.M. of seven observations). Recovery of cyclic AMP in the two fractions was $68.8 \pm 6.9\%$ of that contained in the original synaptosomal material, and recovery of protein was $91.9 \pm 6.1\%$.

Gel filtration of the synaptosomal supernatant fraction gave an elution profile (Fig. 1) that indicated that most of its cyclic AMP was in a higher-molecular-weight form than that of the tissue supernatant, the elution profile of which largely corresponded to that of a sample of cyclic AMP itself. Exposure to phosphodiesterase of the tissue

supernatant fraction after extraction with ether to remove the added isobutylmethylxanthine led to a decrease in its cyclic AMP content to 20% of initial values after 30 min incubation, whereas 60% was retained by the synaptosomal supernatant fraction (Fig. 2); in the continued presence of the xanthine the corresponding values were 40.1% and 58.8% respectively.

Discussion

Routes to the nerve terminals

The cyclic AMP newly formed by stimulation of cerebral tissue has been concluded to be located in limited cellular regions (Daly, 1972; McIlwain, 1974) probably in neuronal perikarya and dendrites (Siggins *et al.*, 1973). These regions would be expected to contribute their soluble contents to the supernatant fraction of Table 3, which indeed was the fraction most enriched in cyclic AMP immediately after stimulation. These findings are consistent with suggestions that cyclic AMP in such experiments is generated postsynaptically after release of neuro-humoral agents (Kakiuchi *et al.*, 1969; Sattin *et al.*, 1975; Daly, 1975).

However, it is also postulated that cyclic AMP acts presynaptically (Robison *et al.*, 1971, pp. 29-30; Wooten *et al.*, 1973; Roth *et al.*, 1975), and in relation to this proposal stimulation of cyclic AMP formation has been sought in isolated synaptosomal fractions (De Bellerocche *et al.*, 1974), but not found. An interpretation can be offered from the present data, for we observed the cyclic AMP in our synaptosomal fraction to undergo a post-stimulation increase (Table 3). This is compatible with the findings of De Bellerocche *et al.* (1974), granted that the cyclic AMP has been generated in part of the tissue not present in the synaptosomal fraction (e.g. the perikarya and dendrites) and subsequently translocated to the nerve terminals. This interpretation is valid even though the synaptosomal fraction may include some resealed membrane material from sources other than nerve terminals (Cotman *et al.*, 1970; Marchbanks, 1975).

To this translocation, intracellular and extracellular routes may contribute (McIlwain, 1976). Cyclic AMP exists extracellularly in the brain (Robison *et al.*, 1971, pp. 142-144; Cramer *et al.*, 1972), but constitutes a small proportion only (2.5%) of the mixture of adenine derivatives released from cerebral tissues on stimulation under the conditions of the present experiments (Pull & McIlwain, 1973). On addition of cyclic AMP to cerebral tissues much more was degraded than was assimilated (Pull & McIlwain, 1977), so making less likely (but not strictly excluding) its translocation by extracellular routes. The intracellular pathways from postsynaptic to presynaptic regions in the mammalian brain are likely to involve

axonal and/or dendritic transport for distances of some 0.02–1 mm (see McIlwain, 1977); by cytoplasmic transport at 5 μ m/s (the 'fast' rate; Ochs, 1974; Lubinska, 1975) these can be covered within the few minutes allowed for redistribution in the experiments of Table 4; a detailed appraisal has been given elsewhere (McIlwain, 1977).

Evidence for the occurrence of cytoplasmic transport in isolated cerebral tissues under conditions similar to those of the present experiments has been given by Jones & McIlwain (1971) and by White (1976). In cat motor neurons *in vivo* and in rabbit neocortex and geniculate, [^{3} H]adenine nucleotides have been observed by radioautography to undergo axonal and dendritic transport (Schubert & Kreutzberg, 1974, 1975). In these experiments, trichloroacetic acid extraction suggested that much of the migrating ^{3} H-labelled-derivatives were in oligonucleotide rather than polynucleotide form, but did not further specify the nature or state of combination of the nucleotide. The major readily labelled trichloroacetic acid-extracted adenine oligonucleotide of normal cerebral tissue is ATP (Santos *et al.*, 1968; Kuroda & McIlwain, 1973), but the observations quoted are consistent with the travelling component including also cyclic AMP.

Combined cyclic AMP

Most of the increased cyclic AMP induced by electrical stimulation was found by centrifugal separation to be free in the general cytoplasmic fraction of the tissue. There was in that fraction a small component only of greater molecular size. By contrast, the soluble cyclic AMP-containing material obtained from the synaptosomal fraction was almost entirely separable from free cyclic AMP and was shown by gel filtration to be appreciably larger in molecular size (Fig. 1). The separations were necessarily associated with some loss of cyclic AMP, as noted in the text, and by making maximal allowance for this loss we conclude that 60–65% of the cyclic AMP of the soluble synaptosomal cyclic AMP is associated with material of higher molecular weight, from which it is separated by trichloroacetic acid. The association hindered the action of phosphodiesterase in the experiments of Fig. 2, a phenomenon noted in a protein kinase-complexed cyclic AMP (O'Dea *et al.*, 1971).

The protein binding of cyclic AMP is considered in many biological systems to be important to its roles of metabolic regulation (Robison *et al.*, 1971, pp. 195–213; Jungmann *et al.*, 1975; Terasaki & Brooker, 1977). There is, however, a further category of protein binding that is likely to be of importance in the present translocation experiments, for certain mechanisms proposed for the movement of substances along nerve fibres (Ochs, 1974) invoke

'transport filaments' that combine with substances undergoing transport and also with the microtubules or neurofilaments of the neuron. Cyclic AMP promotes the cytoplasmic assembly of fibrous proteins, including the microtubules involved in cytoplasmic transport (Lagnado & Tan, 1974; Seitel *et al.*, 1977). If attachment to such entities were to be the basis for cyclic AMP binding in the present experiments, understanding would be afforded of how it is that the cyclic AMP that moves away from its point of production is bound to cell constituents of larger molecular size; this need not exclude the nucleotide from participation in metabolic regulation.

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