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Adenylate and guanylate cyclases in the cerebellum

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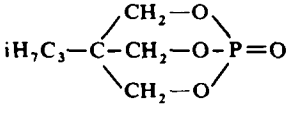
Intracerebroventricular injection of 4-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2,2,2]octane (IPTBO), of caffeine, and of *O*-3,3-dimethylbut-2-yl methylphosphonofluoridate (soman) led to changes in the levels of cyclic AMP and cyclic GMP in the mouse brain [1].



IPTBO

A speculative scheme, in which cyclic AMP was implicated in the inhibitory action of GABA and cyclic GMP was implicated in the excitatory action of ACh, was used to explain the observations and to provide a possible basis for further study. The scheme would suggest that the adenylate cyclase (EC 4.6.1.1) activity and the guanylate cyclase (EC 4.6.1.2) activity in the cerebellum, where the changes were most marked, should be activated by GABA and ACh, respectively. Thus, the effects of GABA and ACh on the adenylate and guanylate cyclase activities in cerebellar homogenates are now reported, together with the effects of some putative GABA antagonists and some anticonvulsants on the modified activities.

Table 1. Enhancement of the basal rates of adenylate cyclase and guanylate cyclase by GABA and ACh respectively

Adenylate cyclase		Guanylate cyclase	
Concentration of GABA (M)	Enzyme activity† (%)	Concentration of ACh (M)	Enzyme activity† (%)
0	100* (5)	0	100
10^{-7}	100 ± 3 (5)	10^{-7}	100 ± 3 (5)
$5\cdot10^{-7}$	110 ± 3 (5)	$5\cdot10^{-7}$	110 ± 3 (5)
10^{-6}	120 ± 4 (5)	10^{-6}	115 ± 3 (5)
$5\cdot10^{-6}$	135 ± 4 (5)	$5\cdot10^{-6}$	130 ± 4 (5)
10^{-5}	145 ± 4 (5)	10^{-5}	140 ± 4 (5)
$5\cdot10^{-5}$	150 ± 5 (5)	$5\cdot10^{-5}$	150 ± 4 (5)
10^{-4}	150 ± 5 (5)	10^{-4}	150 ± 4 (5)
$5\cdot10^{-4}$	140 ± 7 (5)	$5\cdot10^{-4}$	—
10^{-3}	130 ± 8 (5)	10^{-3}	140 ± 6 (5)

* Normal levels (100 per cent) — adenylate cyclase 10–20 pmoles cAMP/assay
≡ 0.5–1.0 μmoles cAMP/mg cerebellum/3 min.
— guanylate cyclase 0.5–1.0 pmoles cGMP/assay
≡ 25–50 nmoles cGMP/mg cerebellum/3 min.

† Percentage of unactivated rate ± S.E.M. Figures in parentheses are number of determinations.

Materials. Cyclic AMP, cyclic GMP, ATP and GTP were supplied by Sigma (London) Chemical Co., Kingston-upon Thames, Surrey, UK. [8-^{3}H]Adenosine 3′,5′-monophosphate (ammonium salt, 27 Ci/mmole) and the 'Radioimmune Assay' kit for cyclic GMP were supplied by The Radiochemical Centre, Amersham, Bucks., UK. Pentobarbitone was obtained from Abbot Laboratories Ltd., Queenborough, Kent, UK. *N*-Methyl bicuculline and picrotoxin were gifts from Dr. J. F. Collins, City of London Polytechnic, while diazepam was donated by Roche Products Ltd., Welwyn Garden City, Herts, UK. Cyclic AMP binding protein and the remaining chemicals, AR-grade where possible, were purchased from BDH Chemicals, Poole, Dorset, UK. IPTBO was prepared by Dr. I. Lawston, Chemical Defence Establishment.

Enzyme preparation. The brains of adult Wistar rats (Porton strain), which had been killed by cervical dislocation, were rapidly removed and cooled on ice. All further manipulations were carried out at 4°. The cerebellar tissue was homogenised in 5 vol. Tris buffer (pH 7.5, 50 mM) containing Mg^{2+}, theophylline, dithiothreitol and EGTA, all at 1 mM concentration. The homogenate was centrifuged at 1000 *g* for 10 min to remove cell debris; the supernatant was further centrifuged at 10,000 g for 30 min and the resultant pellet was resuspended in the same Tris buffer (1:10 volumes based on original weight) for use in the enzyme assays.

Assay of cyclase activity. Tubes (1 × 6 cm) containing 100 μl of 10 mM ATP, or 10 mM GTP, 400 μl of the Tris buffer and 100 μl of the enzyme suspension were incubated for 3 min at 30°. The tubes were then placed in a boiling water bath for 3 min to terminate the enzyme reaction, before being centrifuged at 1000 *g* for 2 min. Aliquots (100 μl) of the supernatants were used for the assay of cyclic nucleotides.

Cyclic AMP was assayed by a modification of the method of Gilman [2] using cyclic AMP binding protein from adrenal cortex and Tris-EDTA buffer at pH 7.5 [3]. A calibration curve (0 to 25 pmoles cyclic AMP) was prepared with each set of analyses.

The radio-immune assay [4] was used for cyclic GMP.

The effects of the modifying compounds on the enzyme activities were determined by adding the compound (in 10μl of solution) to the enzyme incubations to give final concentrations of 100 nM to 1 mM.

Conflicting evidence, some direct but most indirect, for the activation of adenylate cyclase by GABA and for the activation of guanylate cyclase by ACh in various tissues, including cerebellar slices, has been discussed [5]. Further evidence for the apparent activation of the respective enzymes by GABA and ACh has now been obtained using crude enzyme preparations from the cerebellum and this activation has been shown to be concentration-dependent with respect to GABA and ACh (Table 1).

Furthermore, compounds which can antagonise the action of GABA, namely IPTBO [6], *N*-methyl bicuculline [7] and picrotoxin [8], inhibited the GABA-activation of adenylate

Table 2. Effects of drugs on GABA-activated adenylate cyclase and ACh activated guanylate cyclase from the cerebellum

Compound	Concentration (M)	Effect: Adenylate cyclase activity	Effect: Guanylate cyclase activity
GABA		Concentration-dependent acceleration (Table 1)	None
ACh		None	Concentration-dependent acceleration (Table 1)
IPTBO*	10^{-7}	100 ± 10† (3)	None
	$5\cdot10^{-7}$	92 ± 10† (3)	
	10^{-6}	73 ± 10† (3)	
	$5\cdot10^{-6}$	42 ± 10† (3)	
	10^{-5}	10 ± 5† (3)	
N-Methyl bicuculline*	10^{-7}	91 ± 5† (4)	None
	10^{-6}	66 ± 6† (4)	
	10^{-5}	52 ± 4† (4)	
	10^{-4}	26 ± 5† (4)	
	10^{-3}	6 ± 3† (4)	
Picrotoxin*	$4\cdot10^{-6}$	92 ± 8† (3)	None
	$2\cdot10^{-5}$	76 ± 7† (3)	
	$5\cdot10^{-5}$	51 ± 7† (3)	
	10^{-4}	27 ± 5† (3)	
Diazepam	10^{-8}	100 ± 3‡ (3)	None
	10^{-7}	123 ± 5‡ (3)	
	10^{-6}	152 ± 7‡ (3)	
	10^{-5}	151 ± 7‡ (3)	
Pentobarbitone	10^{-7}	112 ± 5‡ (3)	None
	10^{-6}	126 ± 5‡ (3)	
	10^{-5}	151 ± 5‡ (3)	
	10^{-4}	148 ± 5‡ (3)	

* No effect on unactivated rate.

† Percentage of GABA-activated rate (10 μM GABA) minus unactivated rate ± S.E.M. Figures in parentheses are numbers of determinations.

‡ Percentage of unactivated rate ± S.E.M. Figures in parentheses are indicates numbers of determinations.

cyclase (Table 2)—IPTBO and *N*-methyl bicuculline were equipotent and picrotoxin was an order of magnitude less potent—and compounds capable of reversing the effects of GABA antagonists in certain tests [9], diazepam and pentobarbitone, raised the level of cyclic AMP production at 1 μM concentration to that produced by 5 μM GABA. The convulsants had no obvious effects on the basal rate of the enzyme, and the anticonvulsants had no effects on the activation by GABA. None of the compounds affected either the basal rate of the ACh-activated rate of the guanylate cyclase. The mechanisms involved are not yet known but are being studied: for example, the elevation of the basal rate of adenylate cyclase by the anticonvulsants may be caused by a GABA-mimetic action, by the release of endogenous GABA, or, perhaps, by an action on the protein inhibitor of high-affinity binding of GABA [10,11]. However, the results are consistent with the possible function of cyclic AMP as a secondary messenger for GABA in the cerebellum, and of cyclic GMP as a secondary messenger for ACh.

The GABA-activated adenylate cyclase might provide a basis for a screening method for potential convulsant and anticonvulsant compounds. However, it must be noted that the cerebellum receives fibres from many parts of the brain [12], and drugs affecting these parts could as a consequence affect the output from the cerebellum; hence such convulsant or anticonvulsant compounds may have no direct effect on the GABA-activated enzyme.

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Rotenone and oligomycin-like action of trimebutine on liver mitochondria

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In a previous paper it was shown that papaverine and related compounds inhibit liver mitochondria respiration by a rotenone-like mechanism [1]. This finding raised the question as to whether the biochemical mechanism of some spasmolytic agents might involve significant alterations of mitochondrial functions. We have now reexamined the problem by using trimebutine (phenyl-2-dimethyl-amino-2-n, butanol-trimethoxy-3,4,5 benzoic acid), a drug exhibiting spasmolytic activity, which selectively inhibits the "tonic phase" of intestinal smooth muscle contraction induced by acetylcholine or histamine. Since this compound of the smooth muscle contraction seems to be strictly dependent on aerobic energy [1], an insight into the possible interference of the drug with mitochondrial energy dependent processes can provide useful indications for unravelling the mechanism of action of the drug. In the present communication is reported that trimebutine exhibits on liver mitochondria both a rotenone-like and oligomycin-like action which might be relevant for explaining its pharmacological effects [2].

Methods and results. Rat liver mitochondria were isolated according to Schneider [3]. The amount of protein was determined by the biuret method, as described by Gornall [4]. Oxygen uptake was measured polarographically with a Clark electrode coupled to a Perkin Elmer 56 Recorder. ATPase activity was estimated from pH records [5].

Figure 1A shows that in the presence of NAD-dependent substrates (glutamate plus malate or hydroxybutyrate) trimebutine inhibited oxygen uptake stimulated either by ADP (state 3) or FCCP (uncoupled state). This inhibition is concentration-dependent and it is almost complete at 0.15 mM-trimebutine. The inhibition of oxygen uptake induced by trimebutine was not affected by addition of NAD^+ (results not shown), while it was overcome by menadione (K_3) (Fig. 2). With succinate as substrate trimebutine only slightly affected the rate of respiration in state 4 (results not reported), as well as the rate of respiration released by FCCP (Fig. 1B). These findings clearly indicate that trimebutine exhibits a typical rotenone-like action. The small inhibition of succinate respiration in state 4 or in the presence of FCCP (Fig. 1B) can be interpreted as a slight inhibition of succinic dehydrogenase itself, or of the electron flow through the respiratory chain beyond the rotenone sensitive site.

On the other hand trimebutine inhibited state 3 respiration (Fig. 1B) as well as state 4 → 3 transition induced by ADP with succinate as substrate; FCCP completely relieved such an inhibition (Fig. 3A). This would indicate that trimebutine inhibits ADP phosphorylation with an oligomycin-like mechanism. This assumption is further supported by the results of Fig. 3, which show that trimebutine, as well as oligomycin, did not affect the release of respiration by FCCP. Figure 3B shows that trimebutine, as well as oligomycin, did not affect the release of respiration induced by added Ca^{2+}.

Finally, the oligomycin-like action of trimebutine is also shown by the inhibition of ATP hydrolysis evoked by the uncoupler FCCP (Fig. 4).

Discussion. The results reported in the present paper show