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CYCLIC NUCLEOTIDE CONCENTRATIONS IN THE BRAINS OF MICE TREATED WITH THE CONVULSANT BICYCLIC ORGANOPHOSPHATE, 4-ISOPROPYL-2,6,7-TRIOXA-1-PHOSPHABICYCLO[2,2,2] OCTANE

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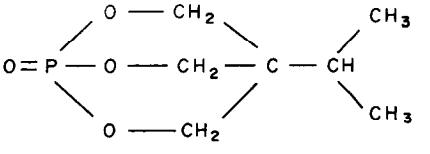
Abstract—The possible involvement of brain cyclic nucleotides in the mechanism of toxicity of 4-alkyl-2,6,7-trioxa-1-phosphabicyclo[2,2,2]octanes—convulsant bicyclic organophosphates without anticholinesterase properties—has been studied by examining the effects of the intracerebroventricular application of the 4-isopropyl derivative (IPTBO) on the concentrations of cyclic AMP and cyclic GMP in three subsections of the brain of the mouse. IPTBO and caffeine, a convulsant phosphodiesterase inhibitor included in the study for comparison, produced convulsions accompanied by decreases in the cyclic AMP concentrations in the cortex, the subcortex and the cerebellum, and increases in the cyclic GMP concentrations in the subcortex and the cerebellum. Pretreatment with pentobarbitone (intraperitoneally) protected against convulsions and the concomitant changes in the cyclic nucleotides produced by IPTBO. In contrast, Soman, a potent convulsant anticholinesterase, produced a convulsive state with increased levels of cyclic GMP and cyclic AMP in the cerebellum and the cortex.

A speculative scheme is proposed (i) to explain the results, which do not appear to support the direct involvement of the inhibition of the brain cyclic nucleotide phosphodiesterase in the mechanism of toxicity of the bicyclic organophosphates or caffeine, and (ii) to provide some basis for further study.

Alkyl derivatives of 2,6,7-trioxa-1-phosphabicyclo-[2,2,2]octane are highly toxic organophosphates which are not anticholinesterases [1–3]. It was suggested that interference with the physiological levels of cyclic AMP was implicated in the mechanism of their toxicity [4], especially since the symptoms of poisoning were similar to those of poisoning by the methyl xanthines, such as caffeine and theophylline [5], compounds which can modify both the activity of adenylate cyclase (EC 4.6.1.1)—the enzyme responsible for the synthesis of cyclic AMP—and the activity of cyclic AMP phosphodiesterase (EC 3.1.4.17)—the enzyme responsible for the removal of cyclic AMP [6–8]. *In vitro* experiments with cyclic AMP as the substrate [4] showed that while the inhibition of the high-K_m form of the phosphodiesterase is unlikely to be involved in the toxic action of these bicyclic compounds, the inhibition of the low-K_m form of the enzyme cannot be entirely excluded. Since this high affinity (low-K_m) form of the enzyme is likely to be the more important for the regulation of the physiological levels of cyclic AMP [9], and indeed cyclic GMP, further studies were initiated to determine whether the bicyclic phosphates would upset the normal physiological levels of both these cyclic nucleotides under *in vivo* conditions. Inhibition of the phosphodiesterase should raise the concentrations of the cyclic nucleotides.

The effects of the direct injection of one of the most toxic of the bicyclic organophosphates, 4-isopropyl-2,6,7 - trioxa - 1 - phosphabicyclo[2,2,2]octane - 1 - oxide (IPTBO) [1, 3], into the lateral ventricle of the



mouse brain on the concentrations of cyclic AMP and cyclic GMP in broadly-defined subsections of the brain (cortex, subcortex and cerebellum) were determined, and compared with those of similar treatment with caffeine. In some experiments the convulsions produced by IPTBO and caffeine were controlled by pretreatment (intraperitoneally) with pentobarbitone. A potent, convulsive anticholinesterase, soman (0–3,3-dimethylbut-2-yl methylphosphonofluoridate) [10], was included for comparison.

MATERIALS AND METHODS

Adenosine 3′,5′-monophosphoric acid was supplied by Sigma Chemical Company. [8-^{3}H] Adenosine 3′,5′-monophosphate (ammonium salt, 27 Ci/mmol) and the 'Radio-Immune Assay' kit for cyclic GMP were supplied by the Radiochemical Centre, Amersham. IPTBO and soman were synthesised at the Chemical Defence Establishment. Cyclic AMP binding protein from adrenal cortex was obtained from British Drug Houses, as were all other chemicals which were either AR or scintillation grade.

Animals and treatment

Groups of 10 Porton-strain male albino mice (20–25 g) were used. Some groups were treated with IPTBO, soman or caffeine nitrate by direct injection into the lateral ventricle [11]. Soman (2.2 μg/mouse) and caffeine nitrate (750 μg/mouse) were administered in 10 μl of saline (0.9%, w/v), and IPTBO (480 ng/mouse) in 10 μl of 10% (w/v) propylene glycol. The doses were previously determined [12] convulsive doses, ED99. At the onset of convulsions the animals were sacrificed by cervical dislocation and placed in an ethanol/'dry ice' freezing mixture for 15 s. The brains were removed as quickly as possible and immediately frozen.

Other groups of animals were (i) pretreated intraperitoneally (i.p.) with sodium pentobarbitone (25 mg/kg) 10 min before, and sacrificed 1 min after, the intracerebroventricular application (i.v.) of IPTBO, (ii) injected (i.v.) only with 10 μl of the solvents and sacrificed 1 min later, (iii) treated with pentobarbitone (25 mg/kg, i.p.) and sacrificed 11 min later, or (iv) sacrificed with no treatment.

Determination of cyclic nucleotides

The cortex, subcortex and cerebellum of each of the frozen brains were dissected [13], weighed and kept frozen until individually homogenised in ice-cold Tris–HCl buffer (1 ml/sample: 0.05 M; pH 7.5) containing EDTA (4 mM). The homogenates were centrifuged at 10,000*g* and 4° for 15 min, and aliquots (50 μl) of the supernatant used for the assay of the nucleotides.

Cyclic AMP was assayed by a modification of the method of Gilman [14] using cyclic AMP binding protein from adrenal cortex and Tris–EDTA buffer (pH 7.5) [15]. A calibration curve (0–25 pmol of cyclic AMP) was prepared with each set of analyses.

The 'radio-immune' assay [16] was used for cyclic GMP.

All the results were analysed statistically using the Student's '*t*' test [12].

RESULTS

The results are presented in Table 1. The handling and injection procedures with the solvents produced no changes in the concentrations of the cyclic nucleotides in any of the three regions of the brain, when compared with those in untreated mice. However, pretreatment with pentobarbitone before injection of solvent increased the cyclic AMP levels in the cerebellum and the cortex but not in the subcortex while having no effects on the cyclic GMP concentrations.

Treatment with IPTBO and caffeine produced convulsions and qualitatively similar effects on the nucleotides: cyclic AMP concentrations were decreased in all three parts of the brain but the decrease in the cerebellum following caffeine treatment was non-significant; the concentration of cyclic GMP was elevated in the cerebellum and in the subcortex but not in the cortex. Pretreatment with pentobarbitone prevented the effects of subsequent treatment with IPTBO. However, the cyclic GMP concentration was increased in the cerebellum by IPTBO even after pretreatment with pentobarbitone but the increase was not so marked as in the absence of pretreatment.

Injection of soman produced a convulsive state with increased levels of cyclic GMP in all three regions of the brain, and increased cyclic AMP concentrations in the cerebellum and cortex, but not in the subcortex.

DISCUSSION

The possibility that the inhibition of the high-affinity form of the cyclic nucleotide phosphodiesterase might account for, or contribute to, the toxicity of the bicyclic organosphosphates such as IPTBO [4] prompted the present study. Direct application into the brain was used to allow rapid inhibition of the diesterase, if such inhibition were involved in the mechanism of toxicity, and to eliminate, or minimise, any peripheral mechanisms which might affect the central, cyclic nucleotide concentrations. It was expected that inhibition of the diesterase would cause an increase in the concentrations of one or both of the cyclic nucleotides. Indeed there were increases in the cyclic GMP levels in two regions of the brain after treatment with IPTBO; however, cyclic AMP concentrations were correspondingly decreased. The increases in the cyclic GMP levels could result from inhibition of the diesterase but not the decreases

Table 1. Effects of treatment with pentobarbitone (i.p.), IPTBO (i.v.), caffeine (i.v.), and soman (i.v.) on the concentrations of cyclic AMP and cyclic GMP in the cerebellar, cortical and subcortical regions of the mouse brain

Treatment	Concentrations of cyclic nucleotides (mean ± S.E.M.; pmol/g)					
	Cerebellum		Cortex		Subcortex	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
None	1291 ± 88	42.2 ± 2.9	737 ± 42	24.2 ± 2.5	1114 ± 69	24.8 ± 2.8
Pentobarbitone (i.p.)	1907 ± 129§	39.1 ± 3.0*	1022 ± 41§	24.1 ± 2.7*	1182 ± 101*	35.5 ± 2.1†
IPTBO (l.v.)	669 ± 49§	69.6 ± 11.2†	510 ± 25§	25.9 ± 1.7*	524 ± 27§	83.4 ± 14§
Caffeine (l.v.)	1099 ± 46*	103 ± 9.4§	575 ± 31‡	22.9 ± 1.9*	735 ± 38‡	119 ± 43§
Soman (l.v.)	1954 ± 196§	122 ± 8.4§	1260 ± 72§	43.4 ± 1.7§	1227 ± 126*	64.1 ± 2.2§
IPTBO (l.v.) and pentobarbitone (i.p.)	1482 ± 145*	57.4 ± 3.6†	807 ± 54*	31.4 ± 2.7*	930 ± 88*	34.0 ± 3.2*

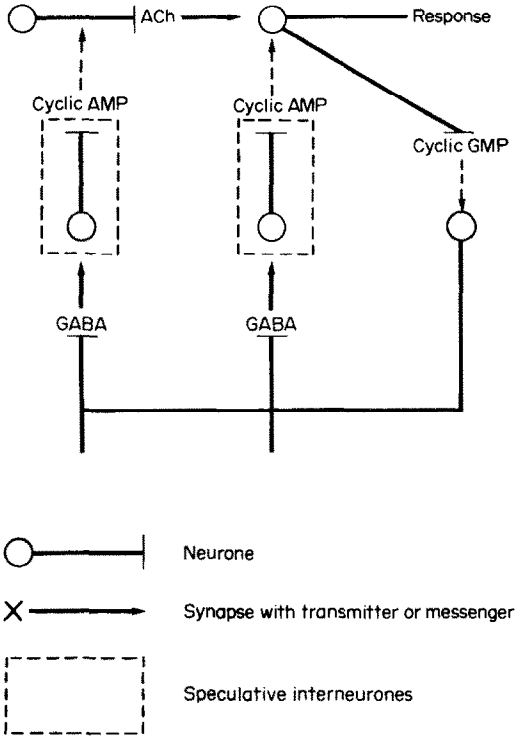
*—Non-significant, $P \geqslant 0.1$
†—$P \leqslant 0.01$
‡—$P \leqslant 0.005$
§—$P \leqslant 0.001$
} Compared with the corresponding control.

in the cyclic AMP levels. Furthermore, the pretreatment with pentobarbitone, which was used to prevent convulsions without interfering with enzyme inhibition, produced an increase in cyclic AMP concentrations. The present results would not therefore support a mechanism for the toxicity of the bicyclic organophosphates directly involving inhibition of the enzyme necessary for the destruction of the cyclic nucleotides. Even in poisoning by caffeine the significance of inhibition of the brain phosphodiesterase would not seem to be great and an unknown alternative mechanism is indicated. This is in accord with the recently reported correlation between the increased locomotive activity and increased cyclic GMP/cyclic AMP ratio in the brains of mice treated with caffeine (i.p.) [17].

Clearly changes in the proportions of the two cyclic nucleotides are somehow implicated in the convulsive activities of the bicyclic organophosphates, because not only do these proportions change during poisoning but the changes can be blocked by a pretreatment with pentobarbitone which protects the animals. Cyclic GMP has likely excitatory effects and cyclic AMP has likely inhibitory effects in the brain [18–20]; thus a profound effect of an upset in the normal proportions of these cyclic nucleotides would not be surprising. In the rat, however, subconvulsive and convulsive doses of IPTBO given i.p. raised the cyclic GMP concentrations in the cerebellum but not in the cortex and the subcortex, and had no significant effects on the cyclic AMP levels in any of the brain areas [21]. The differences between these results and those of the present study may be explained by the different species, route of application and method of killing, but further studies are necessary to confirm this. The changes in the cyclic GMP levels following subconvulsive doses of the bicyclic organophosphate suggest that the changes are not due to the convulsive state [21], but even though the effects of the treatment with IPTBO on the cyclic nucleotide concentrations may have no direct neurophysiological significance, it is interesting to interpret the present results in the context of the possible role of the cyclic nucleotides as secondary transmitters. This is especially true because of recent evidence which indicates that the bicyclic organophosphates are GABA antagonists [22].

Cyclic AMP is envisaged as the secondary transmitter released when some GABA-ergic receptors are stimulated. This cyclic AMP modifies the firing of excitatory fibres whose transmitter is acetylcholine which in turn can trigger the release of cyclic GMP. An antagonism of the GABA receptor by IPTBO would reduce the GABA-stimulated release of cyclic AMP resulting in increased firing of the cholinergic fibres and an increase in the release of acetylcholine: this acetylcholine would stimulate the release of cyclic GMP. Thus, a decrease in cyclic AMP and an increase of cyclic GMP would be observed after treatment with IPTBO. Pentobarbitone can reverse the blockade of GABA receptors [23] so preventing the decrease in cyclic AMP and the increase in cyclic GMP levels. Pentobarbitone can also apparently enhance GABA mechanisms that influence acetylcholine sensitive cells [24] and this could account for the increase in cyclic AMP levels observed after pentobarbitone treatment. Thus, pentobarbitone acts, in the present interpretation, as an agonist at the GABA receptors in a similar way to its activity on GABA receptors in frog spinal cord preparations [25]. The smaller decreases in the cyclic AMP concentrations and the greater increases in the cyclic GMP concentrations caused by caffeine when compared with those after IPTBO treatment suggest that, while caffeine probably has some similar GABA-blocking activity, its inhibitory effects on the phosphodiesterase and stimulatory effects on the cyclase enzyme [6–8] modify the manifestation of this activity.

The effects of soman probably involve interference with GABA-ergic mechanisms as well as interference with cholinergic mechanisms. Inhibition of acetylcholinesterase would raise acetylcholine levels and cause stimulation of cyclic GMP release, thus accounting for the observed effect on this nucleotide of treatment with soman. The concomitant increase of cyclic AMP could be brought about by a compensating 'feedback' mechanism mediated through cyclic GMP. The possibility that stimulation of the muscarinic acetylcholine receptor leads to the release of cyclic GMP [26, 27] has been disputed [21].



Thus, a scheme (represented diagrammatically in Fig. 1) is proposed to explain the observations in studies with a new class of convulsants which are apparent GABA-blockers: a tentative scheme which is intended to form a basis for, and to stimulate, further studies. It is important to emphasise that while there is evidence to support the above explanations, for example evidence for cyclic GMP being a secondary transmitter for an excitatory transmitter [28–30], for the indirect action of GABA on cyclic GMP through another transmitter [30], and for the possible

involvement of GABA in anticonvulsant activity [31, 32], there is conflicting evidence for cyclic AMP being a secondary transmitter for GABA, varying from no involvement [33] to claims for a decrease in cyclic AMP levels in slices of mouse cerebellum after incubation with GABA [34]. However, it might be possible from comparing the effects of the intracerebroventricular application of GABA-blockers, anticholinesterases and anticonvulsants on the cyclic nucleotides with and without pretreatment to provide further evidence to help in the understanding of the ramifications of the actions of anticonvulsants and the interactions of the inhibitory transmitter GABA with excitatory transmitters.

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