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CENTRAL EFFECTS OF THE PRESERVATIVE, METHYLPARABEN

IN VIVO ACTIVATION OF cAMP-SPECIFIC PHOSPHODIESTERASE AND REDUCTION OF CORTICAL cAMP

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Abstract—The phenolic preservative, methylparaben (MPB), has in the past been demonstrated to harbour definite pharmacological effects. In an attempt to examine the possible central effects of MPB, notably on cyclic nucleotides and cyclic nucleotide phosphodiesterase (PDE; EC 3.1.4.17), rats were orally treated with the drug (0.4% in rat food) for 3 weeks with cortex extracts being used for the various determinations. Three isozymes were identified by DEAE-cellulose anion exchange chromatography, namely the calmodulin/calcium-stimulated form or PDE I (peak I), the cGMP-stimulated form or PDE II (peak II), and an independent form not affected by either calmodulin or cGMP also known as PDE IV (peak III). The presence of MPB induced a significant decrease in cortical cAMP, as well as strongly stimulating the activity of PDE IV (peak III). In addition, a small, yet significant, increase in cGMP levels was observed. Since no increase in cGMP hydrolysis was observed, we conclude that chronic ingestion of MPB induces a preference for cAMP hydrolysis, which was confirmed by the increase in PDE IV (peak III) activity. PDE IV is a membrane-bound, low K_m PDE exhibiting high selectivity for cAMP hydrolysis. While there was an increase in cGMP, we failed to observe an increase in the activity of the cGMP-stimulated PDE (PDE II). These data are discussed with reference to the possible membrane effects of MPB allowing it to alter both the kinetic properties of PDE IV with the resultant effects on cAMP, as well as a means whereby it may activate guanyl cyclase and increase cGMP.

Methylparaben (MPB†) belongs to the family of hydroxybenzoates, a series of organic compounds produced by esterification of *p*-hydroxybenzoic acid [1] and is often included in a variety of drug dosage forms and beverages as an antibacterial and antifungal agent [2]. Various *in vitro* as well as *in vivo* studies have demonstrated that this commonly used preservative harbors definite pharmacological properties. The methyl and propyl derivatives of hydroxybenzoate have been found to possess powerful vasodilatory effects *in vivo* [3, 4], to inhibit prostaglandin synthesis [5] and to suppress cGMP levels *in vitro* [6]. They have similarly been found to induce nuclear changes by inhibiting the synthesis of DNA and RNA [7]. No studies to date have been done on the possible central effects of MPB, yet the drug is capable of entering the brain [8] and since its enzymatic hydrolysis is not fully functional in the CNS, the preservative may thus accumulate to appreciable levels [3] allowing it, if able, to have definite pharmacological effects within the brain.

Brain tissue is an extremely rich source of phosphodiesterase (PDE) [9] where it is responsible for the intracellular regulation of the cyclic nucleotides, cAMP and cGMP [10]. The enzyme was first found to exist in multiple forms two decades ago [11] and today at least five different isozyme families are now recognized [10]. All or certain of these isozymes may be isolated by ion exchange chromatography [12] depending on the tissue being examined. In the brain, only three forms have been proposed to exist [13] and include a calmodulin/calcium-stimulated form, known as PDE I, with variable affinity for both cAMP and cGMP, a form stimulated by micromolar concentrations of cGMP, known as PDE II, with high K_m for both cyclic nucleotides, and which also responds to calmodulin, and an independent form insensitive to either calmodulin or cGMP, known as PDE IV, and possessing high affinity and specificity for cAMP [12, 13]. A fourth form, termed the cGMP-inhibited form, due to it being inhibited by micromolar concentrations of cGMP [12], known as PDE III, is either absent or has low activity in the brain [12, 14]. The importance of PDE IV in the brain is displayed by observations that specific inhibitors of this isozyme produce definite changes in behaviour and cognition [12, 15], induce neuronal excitability [16] and are capable of producing antidepressant effects [17].

The concentration of the preservative included in injectables and other pharmaceutical dosage forms is between 0.1 and 0.4% [2]. The concentrations described above best constitute the most likely dose of MPB to which the body will be exposed when used as a preservative in a given drug dosage form. MPB has low oral toxicity in rats [4]. We thus selected the maximum dose of 0.4% administered orally in crushed rat chow over a period of 3 weeks, which we hoped would result in sufficient

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† Abbreviations: MPB, methylparaben; PDE, phosphodiesterase; DEAE, diethylaminoethyl; dbcGMP, dibutyryl cGMP.

accumulation of MPB within the brain after oral ingestion without any deleterious effects on the animal.

MATERIALS AND METHODS

Materials. All reagents were of the highest grade commercially available. MPB, calmodulin, cAMP, dibutyryl cGMP and benzamidine were purchased from Sigma. cAMP and cGMP assay kits were purchased from Amersham and [^{3}H]cAMP (sp. act. 52 Ci/mmol) was obtained from New England Nuclear.

Preparation of cortices. Male Wistar rats (220–250 g) were housed four to a cage and maintained in a constant light–dark cycle with constant temperature and free access to food and water. MPB was incorporated into crushed rat chow (0.4%) and administered over a period of 3 weeks.

Immediately after the rats were killed, cortices were dissected on an ice-cooled dissection slab. Each cortex was halved, and of the four rats representing one group, two left halves and two right halves were pooled for cyclic nucleotide determination, while the corresponding halves were processed for PDE separation and assay. Samples for cyclic nucleotide determinations were homogenized in 0.1 M HCl according to a method described by Weller *et al.* [18] and Tovey *et al.* [19]. The samples were frozen in liquid nitrogen at pH 4 until sufficient samples had been obtained to perform a batch assay. Samples for assay of PDE were homogenized in buffer containing 20 mM Tris, 2 mM benzamidine, 2 mM EDTA and 50 mM sodium acetate (pH 6.5).

The crude extract was then filtered through glass wool and sonicated for 4 min at maximum setting. This sonicated supernatant was then centrifuged at 100,000 *g* for 60 min.

Separation of PDE isozymes. The separation and assay of PDE were modifications of those described previously [11, 20]. Whatman DE 52 DEAE-cellulose anion exchange resin was equilibrated in 300 mL 1.5 M sodium acetate (pH 6.5) and packed in a Pharmacia 400 × 20 mm glass column. The resin was then washed with sodium acetate 1.5 M (pH 6.5) until the column assumed a constant height followed by washing with four bed-volumes of 50 mM sodium acetate (pH 6.5). After adjusting the pH of the sample to 6.5, it was layered onto the column and washed with two bed-volumes of 50 mM sodium acetate (pH 6.5). The PDE isozymes were then eluted from the column by applying an isocratic salt gradient of 400, 500, 700, 900 and 1100 mM sodium acetate (pH 6.5) and collecting 3 mL fractions at a drop-speed of 1 mL/min.

Assay and identification of eluted PDE isozymes. PDE activity for each fraction was determined using the two-step isotopic assay described previously [11] in a reaction volume of 400 μL containing 0.04 M Tris–HCl (pH 8.0), 0.01 M $MgCl_2$, 0.004 M 2-mercaptoethanol, 20 μg of eluate protein, 1 μM cAMP as substrate and [^{3}H]cAMP (200,000 cpm) as tracer. Identification of the various eluting peaks was performed using 2.5 μg/mL calmodulin with 10 μg $CaCl_2$ and 1.25 μM dibutyryl cGMP (dbcGMP) preincubated in the reaction medium for 10 min.

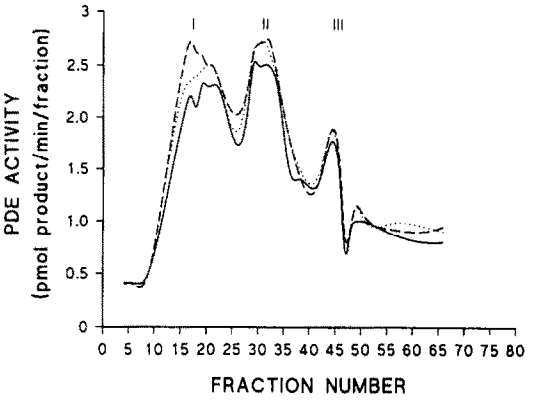

Fig. 1. Resolution profiles of cyclic nucleotide phosphodiesterase activities from rat cerebral cortex. Fractions were assayed with 1 μM cAMP (control; ——), 1 μM cAMP plus calmodulin (2.5 μg/mL) with $CaCl_2$ (10 μg; – – – –) and 1 μM cAMP with 1.25 μM dibutyryl cGMP (· · · · · ·). Each point used to generate these curves was the average of duplicate enzyme activities (pmol product/min/20 μg eluate protein) for the respective groups.

The reaction was initiated with the addition of substrate. For direct comparisons, the activity of the various PDE peaks (pmol product/min/20 μg protein) were expressed as the area under the curve using the Scientific Figure Processing Programme (Fig. P; Fig. P Software Corporation, Durham, NC, U.S.A.). Statistical analysis was performed using the Mann–Whitney U test for non-parametric data.

Cyclic nucleotide assay. cAMP and cGMP were assayed using the radioimmunoassay methods described by Amersham International. The detection limits for the cAMP and cGMP assays were 0.05 and 0.04 pmol, respectively. Samples were thawed and their pH adjusted to 7.5 ± 0.5 immediately prior to assaying. Data are expressed as the mean ± SD.

Protein determination. Protein determination was performed using the method of Lowry *et al.* [21] as modified by Miller [22].

RESULTS

Identifiction of rat cortical PDE isozymes

PDE from rat cortical extracts were resolved into three distinctly eluting peaks by DEAE-cellulose chromatography. A representative elution profile for control untreated animals, and that stimulated by calmodulin and dbcGMP are presented in Fig. 1. Peak I, identified as PDE I, eluted at low ionic strength (fractions 10–27) and was sensitive to calmodulin/$CaCl_2$ stimulation. Peak II, identified as PDE II, was sensitive to both dbcGMP and calmodulin (fractions 27–42). Since stimulation with cGMP is transient due to itself being hydrolysed by PDE [9], the non-hydrolysable analogue, dbcGMP, which has been found to stimulate PDE to a similar extent as does cGMP [23], was used in this study and found to be a more effective stimulant of peak II than cGMP (data not shown). Peak III, identified as PDE IV, eluting at high ionic strength (fractions

Table 1. The effect of chronic methylparaben treatment on rat cortical cyclic nucleotides

Treatment	N (groups of four rats)	cAMP (pmol/incubation tube) Mean ± SD	(Median)	cGMP (pmol/incubation tube) Mean ± SD	(Median)
Control	5	6.990 ± 0.176	(7.05)	0.344 ± 0.011	(0.34)
MPB	5	5.169 ± 0.514*	(5.28)	0.376 ± 0.017†	(0.38)

Significantly different from controls: *P = 0.01; †P = 0.03 (Mann–Whitney U test).

Table 2. The effect of chronic MPB treatment on rat cortical PDE isozyme activity

Treatment	N (groups of four rats)	PDE peak I (pmol/min/20 μg eluate protein) Mean ± SD	(Median)	PDE peak II (pmol/min/20 μg eluate protein) Mean ± SD	(Median)	PDE peak III (pmol/min/20 μg eluate protein) Mean ± SD	(Median)
Control	5	23.00 ± 2.40	(23.21)	18.77 ± 0.97	(19.32)	12.06 ± 1.39	(12.12)
MPB	5	20.67 ± 3.06	(21.49)	19.10 ± 1.71	(18.56)	19.87 ± 2.36	(19.21)*

Enzyme activity data are presented as the total area, equivalent to the total activity, under each peak. Areas were calculated by using the Scientific Figure Processing Programme (Fig. P).
Significantly different from controls: *P = 0.01 (Mann–Whitney U test).

42–55), was insensitive to both calmodulin and dbcGMP (Fig. 1).

Despite the fact that fractions 42–55 appeared to consist of two co-migrating peaks, their pharmacological characteristics with regard to their response to calmodulin and dbcGMP, were similar, and we therefore concluded that this was an artifact caused by the collection of fractions. Furthermore, neither of the two peaks were inhibited by dbcGMP, thus negating the possibility of this peak being the cGMP-inhibited form.

Chronic treatment of rats with MPB was well tolerated, although there were signs amongst some individual animals of locomotor retardation.

The effect of MPB on cortical cyclic nucleotides

A statistically significant drop in cortical cAMP levels was observed in the treated animals compared to controls (P = 0.01; Mann–Whitney U test, Table 1). An observation which surprised us, and which was contrary to previously published *in vitro* data, was the small, yet significant increase in the cGMP levels observed in the MPB-treated animals (P = 0.03; Mann–Whitney U test, Table 1).

The effect of MPB on the elution profile of cortical PDE isoenzymes

Despite an increase in cGMP in the MPB-treated animals, no increase in the cGMP-stimulated PDE (PDE II/peak II; Table 2) was observed. Similarly, no significant change was discernible regarding PDE I (Peak I; Table 2). However, there was a significant increase in PDE IV (peak III) activity in MPB-treated animals compared to controls (P = 0.01; Mann–Whitney U test, Table 2).

DISCUSSION

The phenolic preservative, MPB, is included in the formulation of a wide variety of pharmaceutical preparations [2]. It has also been found to possess a number of pharmacological effects, some of which have resulted in severe iatrogenic side effects not attributable to the main pharmacological ingredient [3, 4]. Since much is documented regarding the effects of MPB in the periphery [3–7], and also that it has the ability to enter [8] and to accumulate in the brain [3], we have examined the possibility of any central effects, notably on cortical cyclic nucleotide second messengers and on the various isozymes of PDE.

Peak III, identified as PDE IV, appeared very sensitive to the presence of MPB. Characteristically, PDE IV is insensitive to calmodulin or cGMP stimulation, as well as not being inhibited by cGMP [12], and has a high affinity (1 μM) and selectivity for cAMP [12]. As found in previous studies [12–14], we have failed to demonstrate the presence of a fourth isozyme in rat cerebral cortex. Our results demonstrate the stimulatory effects of MPB on rat cortical PDE IV (peak 3; Table 2). Furthermore, cortical cAMP levels were dramatically reduced while those of cGMP were slightly raised above that of control animals (Table 1), indicating a preference for cAMP hydrolysis.

Previous studies have not supported an involvement of PDE or cAMP in MPB action [3, 4]. Unlike the natural antioxidants such as the tocopherols, MPB, like the closely related phenolic antioxidants hydroxyanisol and hydroxytoluene, is devoid of a hydrocarbon "tail" [24]. This property is important to enable the natural antioxidants to align themselves with the lipid bilayer of the membrane without disturbing its integrity and function [24]. Butylated hydroxytoluene and other similar compounds have been shown to exert toxic effects on membranes such as the sarcoplasmic reticulum where it blocks

Ca^{2+},Mg^{2+}-ATPase and calcium transport [24, 25] with a similar effect on intracellular calcium proposed for MPB [26]. The results described in this paper provide support for an MPB effect on cell membranes. PDE IV is associated with membranes in the rat brain [12] such that any perturbation of the membrane structure may result in changing affinity and activity. For example, phospholipase C, a membrane-bound enzyme inducing dynamic changes to the membrane through its hydrolysis of inositol and other phospholipids to the second messengers, diacyglycerol and inositol triphosphate [27], has been demonstrated to increase cAMP–PDE activity [28]. Many of the already documented effects of MPB such as those on calcium transport [25, 26], cGMP suppression [6], as well as studies showing MPB to have no effects on either PDE or on intracellular levels of cAMP or cGMP [4] were performed *in vitro*. Because of the dynamic nature of biological membranes, exposure to MPB for 3 weeks in an intact animal may allow the drug to disrupt cellular membranes inducing the observed effects on PDE and cAMP metabolism. Indeed, guanyl cyclase too, exists in two forms, namely a soluble form stimulated by various paramagnetic molecules such as superoxide and hydroxyl radicals [29] as well as nitric oxide [29, 30], and a membrane-associated form stimulated by the natriuretic peptides [29]. Particulate guanyl cyclase can be regulated by events that induce changes to the surrounding membrane structure, such as the effect of phospholipases on guanyl cyclase activity [29]. Consequently, perturbation of membrane structures by MPB may, in a similar way to that described for activation of the membrane-bound form of PDE, be responsible for increasing the inherent activity of membrane-bound guanyl cyclase and thus increase cGMP. In fact, an alternative possibility that may lead to a decrease in cortical cAMP levels is that this observed increase in cGMP levels may stimulate the cGMP-stimulated PDE (PDE II), leading to increased cAMP hydrolysis. Our results do not support this, however, since the PDE elution profile from MPB-treated animals revealed no change in PDE II (peak 2) activity relative to control, while the increase in cGMP observed (0.344–0.376 pmol) may not be sufficient to induce a change in PDE kinetics *in vivo*.

PDE IV has been implicated in a variety of cerebral functions such as behaviour and cognition [12, 15] and in neuronal excitability [16]. Although chronic exposure to MPB for 3 weeks did not produce any marked changes in behaviour, we did observe random symptoms of slight motor retardation. The clinical implications of this observation is speculative and requires further investigation, however, the central subcellular effects of MPB observed in this paper should be noted when one considers the prevalence of the hydroxybenzoates in medicinal preparations.

In conclusion, these data are supportive of an effect of MPB on cortical PDE activity and cAMP metabolism. The preservative induces an isozyme-specific increase in the low K_m, cAMP-specific PDE IV after chronic *in vivo* exposure with a resultant suppressive effect on cAMP levels. This effect may be attributable to the membrane disruptive actions of phenolic compounds such as MPB. The decrease in cortical cAMP, together with the slight increase in cGMP observed, provides additional support for a preference for cAMP hydrolysis. These data, furthermore, reemphasize the subtle pharmacological effects of the phenolic preservatives, of which MPB is only one, and both investigators and clinicians should be aware of the possible iatrogenic effects of these ubiquitous compounds.

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