Simultaneous Delivery of Valproic Acid and Glycine to the Brain

Deamination of 2-Propylpentylglycinamide by Monoamine Oxidase B

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ABSTRACT

2-Propylpentylglycinamide (2-PPG), a branched aliphatic amine derivative, was found to be readily deaminated by rat liver monoamine oxidase B in vitro and in vivo. The deamination leads to production of 2-propyl-1-pentaldehyde, which can be subsequently converted to valproic acid (VPA), and glycinamide, which is then subsequently converted to glycine. Absorption and biotransformation of a single ip dose of 2-PPG into blood as well as transfer of the drug and its metabolite into the brain were rapid processes. Although VPA (an anticonvulsant) and glycine (an inhibitory neurotransmitter) can be detected in the brain following administration of 2-PPG, its anticonvulsant action cannot be determined. 2-PPG at relatively low doses exhibited distinct tremor effects. Furthermore, 2-PPG appeared to potentiate the convulsant effect induced by pentylenetetrazol.

Index Entries: Monoamine oxidase (MAO); valproic acid; glycinamide; glycine; tremor; convulsant; anticonvulsant; 2-propylpentylglycinamide; milacemide.

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INTRODUCTION

Monoamine oxidase (MAO, EC. 1.4.3.4.) is well-known for its catalytic activities on endogenous arylalkylamine substrates, such as neuronal catecholamines, indolylethylamines, and trace amines (*see* review by Yu, 1986). Interestingly, straight-chain aliphatic amines in the range of 3–18 carbon atoms in length are also found to be deaminated by MAO-B with a high affinity (Blaschko, 1952; von Korff and Wolfe, 1984; Yu, 1989).

Recently, MAO has been found to be involved in the conversion of an antiepileptic prodrug, 2-*n*-pentylaminoacetamide (milacemide™) (de Varebeke et al., 1988). Milacemide can cross the blood–brain barrier and then be oxidized by MAO to form glycinamide, which is subsequently cleaved to glycine. The delivery of glycine is presumably related to the anticonvulsant activity of the milacemide (van Dorsser et al., 1983) and the apparent improvement in learning and memory (Handelmann et al., 1989).

We have recently discovered that 2-propylpentylamine (2-propyl-1-aminopentane) can be deaminated by rat liver MAO and aorta semicarbazide-sensitive amine oxidase; it is converted to 2-propylpentaldehyde and then oxidized to valproic acid (VPA) (Yu and Davis, 1991). VPA has been widely used as an anticonvulsant (*see* review by Chapman et al., 1982) and has been reported to be effective in the treatment of bipolar disorders (McElroy et al., 1988). We were interested, therefore, to determine whether or not 2-PPG could be deaminated by MAO in a manner similar to that for milacemide, and thus act as a prodrug for VPA and glycine. We assumed that such a simultaneous delivery of VPA and glycine to the central nervous system might be useful for enhancing anticonvulsant and other pharmacological effects. In this report we describe the deamination of 2-PPG in vitro, its biotransformation in vivo, and some of its behavioral effects.

MATERIALS AND METHODS

Materials

Male Swiss albino mice (25 g) were used; yeast aldehyde dehydrogenase, β-nicotinamide adenine dinucleotide, homovanillic acid, and horseradish peroxidase were purchased from Sigma (St. Louis, MO); *l*-deprenyl (phenylisopropylmethyl propargylamine HCl) was a gift from J. Knoll (Budapest, Hungary); and clorgyline [*N*-(2,4-dichlorophenoxy-*n*-propyl)-*N*-methylpropargylamine HCl] was obtained from May and Baker Ltd.(Dagenham, UK). All other chemicals were of analytical grade.

Synthesis of 2-PPG

Ethyl N-trifluoroacetylglycinate (100 mmol) in acetonitrile (250 mL) was refluxed for 24 h with powdered potassium tert-butoxide (100 mmol) and 1-bromo-2-propylpentane (110 mmol). After filtration and evaporation of the solvent, the product, ethyl N-(2-propylpentyl)-N-trifluoroacetyl glycinate, was stirred at room temperature for 2 h in $2M$ potassium hydroxide in methanol (250 mL) to remove the protecting group. Following evaporation of the solvent, the residue was acidified with concentrated hydrochloric acid, which was then evaporated. The residue was triturated in methanol and filtered from the insoluble potassium chloride. After removal of the solvent, the partially hydrolyzed product was completely esterified by refluxing in methanolic hydrochloric acid. The product, methyl 2-PPG (identity confirmed by mass spectrometry), was recrystallized from methanol-ether (mp 172–174°C) and then dissolved in methanol (150 mL), treated with liquid ammonia, (30 mL) and allowed to stand at 20°C in a closed flask for 7 d. Rotary evaporation of the solvent at 40°C gave 2-PPG as a viscous pale brown liquid, the mass spectrum of which exhibited the expected major ions at 186 (M^+), 142, 87, and 44.

Preparation of Rat Liver Monoamine Oxidase

The liver mitochondrial fractions were prepared by differential centrifugation as previously described (Yu, 1986). Mitochondrial membrane fragments were obtained by lysing the mitochondria in chilled distilled water followed by centrifugation at 105,000g for 30 min. The membrane preparations were further washed, twice, by suspension in water followed by centrifugation. The resultant pellets were homogenized in water by repeated ultrasonic disruption at 75 W peak envelope power for 5 s several times using a needle-tip probe (Braunsonic 1510, San Francisco, CA, USA).

Rat liver MAO-A and MAO-B were obtained by treatments using selective MAO inhibitors. The mitrochondrial membrane enzyme preparations were incubated with either the MAO-B inhibitor l-deprenyl (1×10^{-6} M) or the MAO-A inhibitor clorgyline (5×10^{-7} M) at room temperature for 30 min.

Assay of Deamination of 2-PPG

A sensitive fluorometric method based on the formation of an intense fluorescence from the reaction of homovanillic acid and the hydrogen peroxide released during the oxidation of the amines (Snyder and Hendley, 1968; Yu and Boulton, 1980) was used for the assay of the deamination of 2-PPG. The mitochondrial MAO preparation (130 μg protein) was incubated at 37°C for 10 min in the presence of the amine substrate in a total volume of 200 μL of 0.05M phosphate buffer (pH 7.5) containing 50 μg of homovanillic acid and 0.82 U of horseradish peroxidase. The fluorescence intensity was measured in a spectrophotofluoro-

meter (Aminco-Bowman, Silver Spring, MD) at an excitation wavelength of 315 nm and an emission wavelength of 425 nm. The conversion of aldehyde products to VPA was carried out, when necessary, by including yeast aldehyde dehydrogenase (1.25 U/assay) and β-NAD (5.2 mM) in the enzyme reaction mixture (Yu and Davis, 1988).

Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard protein.

The kinetic parameters were analyzed according to the method of Wilkinson (1961).

Detection of VPA

VPA was derivatized by reaction with 4-bromomethyl-6,7-dimethoxycoumarin (BMDC) as previously described (Dunges, 1977; Cisse et al., 1981). The derivatized products were separated by high-performance liquid chromatography (HPLC) and detected fluorometrically. For the determination of VPA as a product of the deamination of 2-PPG, caproic acid was added to samples as internal standard (200 μL) of the enzyme reaction mixtures, which were acidified by addition of 50 μL of 2M HCl and vortexed vigorously with 1 mL of ethyl acetate. Following centrifugation at 2000g for 5 min, 50 μL of the organic extract was added to a reaction mixture containing 50 μL of BMDC (0.1 mg/mL acetonitrile), 50 μL of 18-crown-6 (1 mg/mL acetonitrile), and 50 μ of K$_2$CO$_3$ (1 g/mL water). The derivatization was carried out in the dark at 50°C for 30 min. A 20-μL aliquot of the derivatized sample solution was injected into the HPLC using a Waters WISP-710B autosampler and separated on a reverse-phase column (Supelcosil LC-18, particle size 5 μ, 250 $\times$ 4.6 mm id, Bellefonte, PA, USA). For the separation of derivatized VPA and caproic acid, a linear gradient with 60–100% (v/v) aqueous acetonitrile was employed in a Waters solvent-gradient system (Waters, Milford, MA, USA) at a flow rate of 1 mL/min. The acids were detected with a fluorescence detector (Hewlett Packard, HP 1046A, Waldbronn, Germany) at an excitation wavelength of 342 nm and an emission wavelength of 439 nm (equipped with a 370-nm cutoff filter). The peak areas were measured on a Spectra-Physics SP-4290 integrator (Spectra-Physics, San Jose, CA, USA).

Conversion of 2-PPG to VPA In Vivo

2-PPG (200 mg/kg), dissolved in saline containing 5% Tween-80, was administered to the mice via intraperitoneal injection. Brain tissues and blood were collected at different times after injection. The blood was centrifuged immediately to obtain serum, to which caproic acid was added as internal standard. Both mouse-brain tissues and the serum were frozen and stored at −70°C until used (within 3 d). The tissues were homogenized in water 1/2.5, w/v) containing caproic acid (75 μg/ mL). Portions (200 μL) of the supernatants of the homogenate and of the serum were acidified with 50 μL of 2M HCl and extracted with ethyl

acetate; aliquots of these organic solvent extracts were then derivatized and analyzed using the HPLC-fluorometric method described earlier.

Behavioral Studies

For observation of 2-PPG-induced tremor in mice, different doses of ip administration of the drug in saline were assessed. After injection of 2-PPG, five animals at a time were kept in a cabinet (30 × 10 × 35 cm; w/l/h) for observation. The occurrence or absence (all or none) of a tremor effect (i.e., from mild, sporadic shivering to intense and continuous tremors, or death) within a 30-min observation period was recorded. For each experiment at least 15 animals were used at each dose of 2-PPG administered via the different routes.

In the study of the interactions of 2-PPG with the convulsants, such as pentylenetetrazol (a potent glutamate decarboxylase inhibitor) and strychnine (a glycine antagonist) the animals were pretreated with different doses of 2-PPG (over a range of 10–200 mg/kg, ip 100 µL/animal, 15 to 40 animals/dose) for 30 min before challenge with the convulsants pentylenetetrazol (50 mg/kg) and strychnine (0.5 mg/kg) via sc injection at the back of the neck (100 µL/animal). The occurrence and intensity of clonic and tonic seizure episodes, tremor, and mortality of the animals were recorded within 30 min after administration of the convulsants.

RESULTS

Deamination of 2-PPG by MAO In Vitro

When 2-PPG was incubated with rat mitochondrial MAO the formation of hydrogen peroxide was detected. The reactions were linear both with time (for at least 15 min) and with respect to increasing enzyme concentrations.

Identification of VPA as the Deaminated Product of 2-PPG

The formation of VPA from 2-PPG following enzymatic deamination was confirmed by HPLC (*see* Fig. 1). As can be seen from Fig. 1C, only a small amount of VPA could be detected when 2-PPG was incubated with MAO alone. This is because 2-propyl-1-pentaldehyde, the intermediate metabolite of 2-PPG oxidation, can not be derivatized by BMDC, and the washed fractions of rat liver mitochondrial membrane do not contain sufficient amounts of aldehyde dehydrogenase and β-NAD to oxidize the aldehyde to VPA (Yu and Davis, 1988). In the presence of MAO, yeast aldehyde dehydrogenase, and β-NAD cofactor, a significant increase of VPA production occurs (*see* Fig. 1D). Incubation in the absence of 2-PPG substrate (Fig. 1A) or MAO (Fig. 1B) does not yield any detectable VPA.
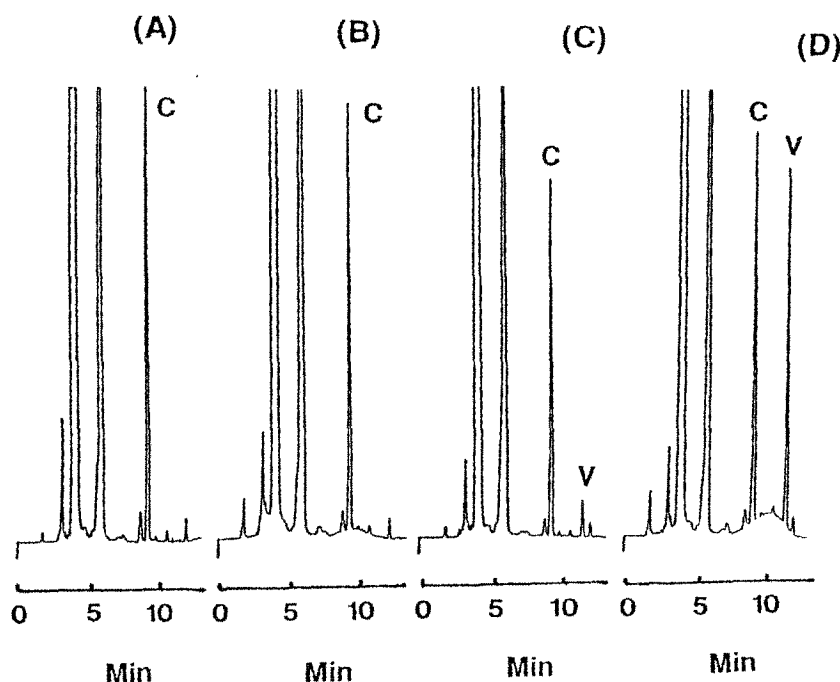
Fig. 1. High-performance liquid chromatographic determination of VPA as a product of deamination of 2-PPG: (A) aldehyde dehydrogenase and β-NAD and 2-PPG included, but without MAO; (B) MAO and aldehyde dehydrogenase and β-NAD included, but without 2-PPG; (C) MAO assay in the absence of yeast aldehyde dehydrogenase and β-NAD; (D) aldehyde dehydrogenase (1.25 U) and β-NAD ($5.2 \times 10^{-3}M$) included, along with 2-PPG and MAO. C—caproic acid as internal standard (retention time, 9.1 min); V—valproic acid (retention time, 11.3 min).

Effect of Specific MAO-A and MAO-B Inhibitors on the Deamination of 2-PPG

Figure 2 shows that the IC_{50} for *l*-deprenyl and clorgyline are $1 \times 10^{-7}M$ and $>1 \times 10^{-4}M$, respectively. That the deamination of 2-PPG is sensitive to *l*-deprenyl rather than to clorgyline indicates that it is predominantly deaminated by MAO-B. This is consistent with our earlier findings regarding the deamination of other straight-chain aliphatic amines (Yu, 1989).

Kinetic Parameters of MAO with Respect to Deamination of 2-PPG

The apparent K_m and V_{max} values for rat liver mitochondrial MAO-B (enzyme pretreated with $5 \times 10^{-7}M$ clorgyline) toward 2-PPG are 78 ± 11 μM and 0.23 ± 0.04 nmol/min/mg protein, respectively. The affinity of
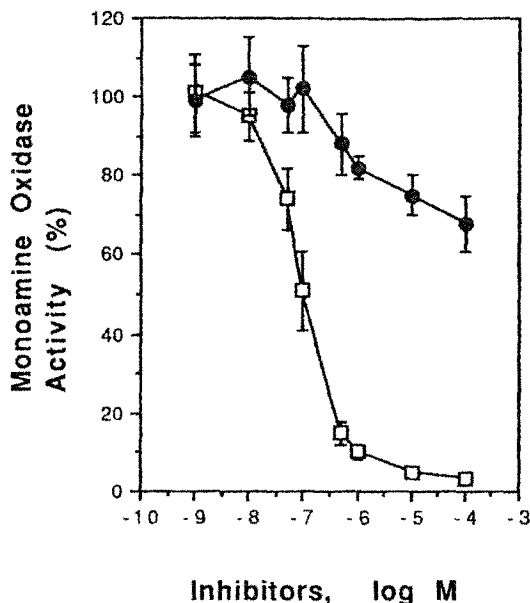
Fig. 2. The effect of the MAO-A inhibitor clorgyline (●) and the MAO-B inhibitor deprenyl (□) on the deamination of 2-PPG by rat liver mitochondrial MAO. Rat liver mitochondrial MAO preparations were preincubated with the inhibitors for 20 min at room temperature. Remaining deaminating activities toward 2-PPG were then assayed. The enzyme activity is expressed as a percentage of the corresponding control samples preincubated without the inhibitor.

2-PPG is relatively lower for both enzymes than for 2-propyl-1-aminopentane [$(CH_3CH_2CH_2)_2CHCH_2NH_2$] (Yu and Davis, 1991) and other straight-chain aliphatic amines (Yu, 1989).

Detection of VPA in Mouse Blood Serum and Brain Tissues After Peripheral Administration of 2-PPG and VPA

VPA, the deaminated product of 2-PPG, was identified by the HPLC-fluorometric method following administration of 2-PPG. Absorption and biotransformation of a single ip dose (100 mg/kg), as revealed by assessment of the drug metabolite VPA in blood serum and the whole brain, were found to be very rapid. The pharmacokinetic analysis of VPA levels after ip injection of 2-PPG is shown in Fig. 3. The highest concentrations of VPA were about 5 μg/mL in serum after 30 min and 0.6 μg/g in the brain after 2 h. The half-lives of VPA in the blood and brain were about 2 and 5 h, respectively. When VPA (100 mg/kg) was administered intra-
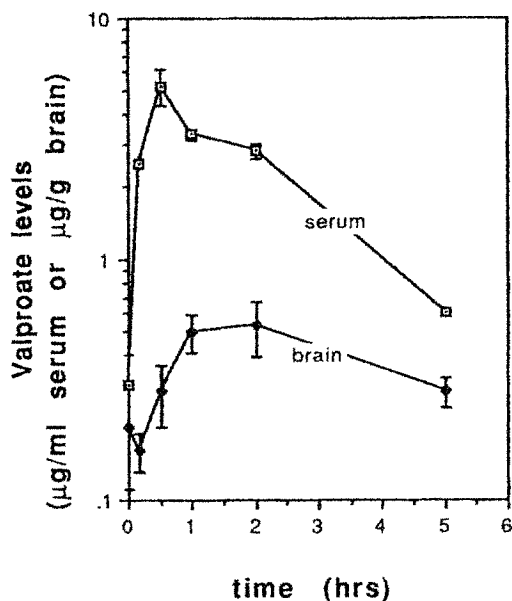
Fig. 3. Detection of VPA levels in mouse brain and blood after a single ip dose of 100 mg/kg of 2-PPG. VPA levels in the blood (□) and brain (♦) following administration of 2-PPG were measured by HPLC-fluorometric method. Values given as means ± SE of at least five animals at each dose.

peritoneally to mice, its maximal levels were 130 μg/mL in blood and 40 μg/g in the brain after 30 min (results not shown). These levels were considerably higher than those reached after administration of 2-PPG. It is interesting to note that VPA levels after administration of VPA decayed much more quickly (Yu and Davis, 1991) than was the case after administration of 2-PPG. After about 2 h the VPA levels were quite similar following administration of either 2-PPG or VPA. This indicates that the conversion rate of 2-PPG to VPA is relatively slower than the rate of elimination of VPA.

Behavioral Effect of 2-PPG in Mice

Since 2-PPG can be oxidized in vivo to produce VPA, we attempted to evaluate whether 2-PPG could exhibit anticonvulsant activity. Unfortunately, any such activity (i.e., after acute injection) proved very difficult to measure, since 2-PPG itself produced intense tremors in mice. Furthermore, we noticed that 2-PPG may also potentiate the convulsions induced by pentylenetetrazol.

As can be seen from Fig. 4, even at relatively low concentrations 2-PPG induces sporadic tremor or shivering responses. The ED_{50} values of this tremor effect (occurrence or absence of tremor within 60 min) was 58 ± 9 mg/kg following the peritoneal administration of the drug. At a dose of 200 mg/kg (ip injection) 2-PPG induced a continuous, intense tremor of the whole body, which lasted for at least 60 min. None of the animals died, however, even at these high doses.
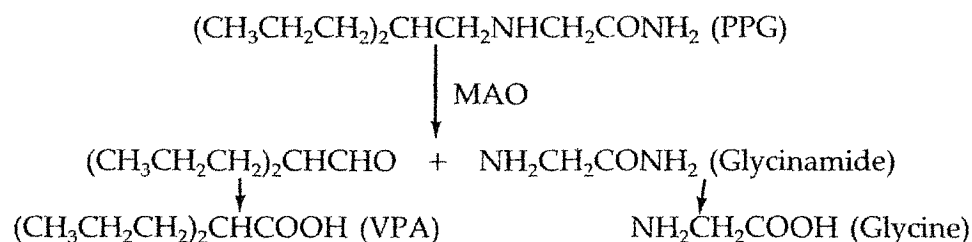
Injections of straight-chain aliphatic amines, such as n-pentylamine and n-octylamine, at relatively high doses (200 mg/kg, ip), did not cause tremor effects.

2-PPG unexpectedly was found to potentiate the effects of several convulsants. Figure 5 shows the results in mice pretreated with different ip doses of 2-PPG for 30 min and then challenged by sc injection of pentylenetetrazol or strychnine. Both convulsion and mortality (LD_{50} = 59 ± 7 mg/kg) induced by pentylenetetrazol increased significantly in the animals pretreated with 2-PPG. Similar potentiation of convulsion has also been observed with respect to convulsion induced by mercaptopropionic acid (unpublished results). The effect of 2-PPG on strychnine-induced convulsion is not quite significant.

DISCUSSION

Our finding that 2-PPG is readily deaminated by rat liver MAO-B further confirms that MAO is capable of metabolizing not only straight-chain and branched aliphatic amines, but also branched aliphatic glycinamides both in vitro and in vivo. The K_m value for the metabolism of 2-PPG by MAO-B is relatively low in comparison with those for many of the catecholamines and indolylamines to these amine oxidases (Yu, 1986).

Deamination of 2-PPG, as indicated below, will lead to the formation of VPA and glycine:

$$(CH_3CH_2CH_2)_2CHCH_2NHCH_2CONH_2 \text{ (PPG)}$$

$$\downarrow \text{ MAO}$$

$$(CH_3CH_2CH_2)_2CHCHO \quad + \quad NH_2CH_2CONH_2 \text{ (Glycinamide)}$$

$$\downarrow \qquad\qquad\qquad\qquad\qquad \downarrow$$

$$(CH_3CH_2CH_2)_2CHCOOH \text{ (VPA)} \qquad\qquad NH_2CH_2COOH \text{ (Glycine)}$$

VPA can be detected in the brain and serum soon after the peripheral administration of 2-PPG; its maximal level after an ip dose of 100 mg/kg is only about 0.6 μg/g brain and 5 μg/mL blood. These levels are probably too low to exert an acute anticonvulsant effect. Using pentylenetetrazol as a convulsant agent, it has been shown, in mice that the ED_{50} doses for VPA were 102 and 149 mg/kg ip and po respectively (Morre et al., 1984).
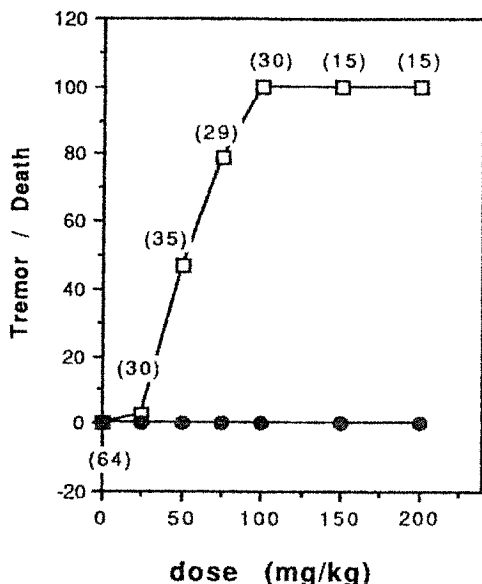
Fig. 4.   Effect of 2-PPG on tremor
and mortality of mice. 2-PPG at differ-
ent doses (10–200 mg/Kg) was adminis-
tered via ip injections. The percent of
occurrence of tremor responses (all or
none, □) or mortality (●) within 30 min
were observed. The number of mice
used for each dosage is indicated in pa-
renthesis marks.

Anticonvulsant activity disappears when the blood VPA levels fall below
70 μg/mL (Chapman et al., 1982). The 100-mg/kg dose of 2-PPG cannot be
increased, because doing so induces severe tremor.

   Following the discovery of the therapeutic properties of VPA many
chemical analogs (*see* review by Chapman et al., 1982) have been tested
for their anticonvulsant activity. Among them, several VPA analogs,
such as 2-butylhexanoic acid, 2-propylheptanoic acid, and 2-propylhex-
anoic acid, have been shown to exhibit more potent anticonvulsant
activity than VPA itself (Chapman et al., 1983). In addition the amide and
various esters of VPA have also been tested (Benoit-Guyod, 1967; Favel et
al., 1973; Musolino et al., 1980). Dipropylacetamide possesses anticon-
vulsant activity in humans, apparently as a consequence of its conversion
to VPA in the gastrointestinal tract (Pisani et al., 1982). The amide itself
seems to possess anticonvulsant activity (Lloyd and Worms, 1981). Pen-
tylglycinamide (milacemide) is a well-known anticonvulsant; replacing
the pentyl group with a propylpentyl group, as described in the present
article, did not potentiate anticonvulsant action: on the contrary, it po-
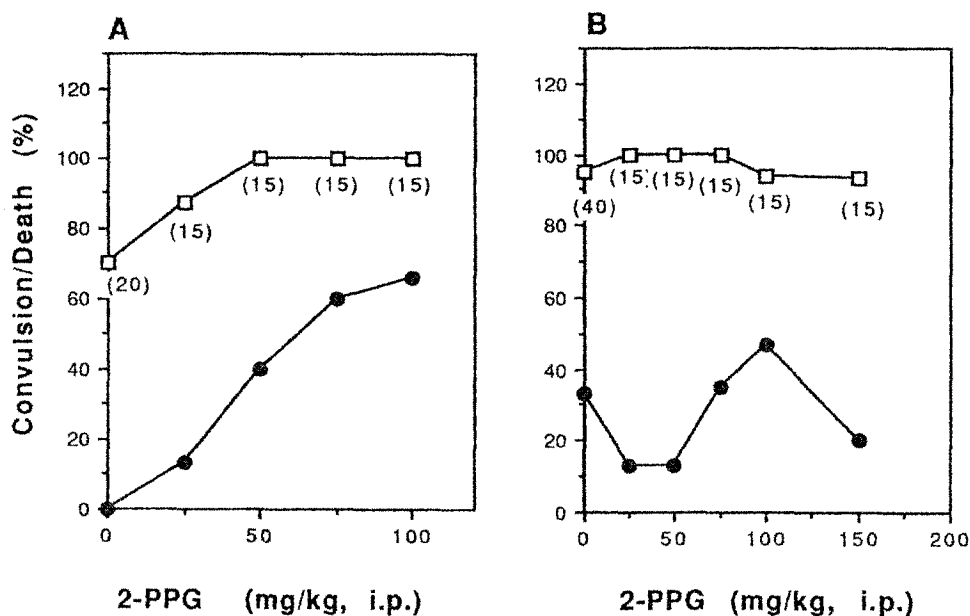tentiated the convulsive action induced by pentylenetetrazol. It is in-

Fig. 5. Potentiation of 2-PPG on convulsions in mice induced by pentylenetetrazol (A) and strychnine (B) Mice were ip injected with different doses of 2-PPG 30 min before administration of convulsants. The challenge doses for pentylenetetrazol and strychnine are 50 and 0.5 mg/kg respectively. The number of mice used for each dosage is indicated in the parenthesis marks. The percent of convulsions (all or none) (□) or mortality (●) within 30 min were observed.

triguing that 2-PPG and VPA should exert opposite behavioral effects. The mechanism of VPA anticonvulsant action, unfortunately, has not yet been well-established (Johnston, 1984).

The finding that 2-PPG can cause tremor is interesting; however, it is unclear whether it is the 2-PPG itself or its immediate metabolite, 2-propyl-1-pentaldehyde, that is responsible for the observed behavioral effects. We have found that the MAO-B inhibitor deprenyl did not block the tremorogenic effect, suggesting that 2-PPG itself, rather than its deaminated aldehyde product, is responsible for the toxic effect. This is supported by an observation of a lack of tremor effect by 2-propyl-1-pentaldehyde (100 mg/kg) (unpublished results). The branched-chain structure of 2-PPG appears to be essential for the induction of the tremor, since straight-chain aliphatic amides, such as n-pentylglycinamide, at the same doses are without such effects. Since 2-PPG is a structural analog of VPA, it is possible that the observed tremor induced by 2-PPG is related to a toxic effect of VPA; it is known that tremor can be a side effect of chronic administration of VPA (Karas et al., 1982). In our test animals, however, acute treatment with VPA at different doses did not induce any obvious tremor. The 2-PPG-induced effect may represent a useful animal

model for the study of a certain kinds of tremors, although its mechanism of action and the nature of involved neuronal systems remained to be elucidated.

ACKNOWLEDGMENT

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