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# THE EFFECTS OF A SERIES OF ω-PHOSPHONIC α-CARBOXYLIC AMINO ACIDS ON ELECTRICALLY EVOKED AND EXCITANT AMINO ACID-INDUCED RESPONSES IN ISOLATED SPINAL CORD PREPARATIONS

R.H. EVANS, A.A. FRANCIS, A.W. JONES, D.A.S. SMITH & J.C. WATKINS*

Departments of Pharmacology and Physiology,* the Medical School, University Walk, Bristol, BS8 1TD

**1** The depressant actions on evoked electrical activity and the excitant amino acid antagonist properties of a range of ω-phosphonic α-carboxylic amino acids have been investigated in the isolated spinal cord preparations of the frog or immature rat.

**2** When tested on dorsal root-evoked ventral root potentials, members of the homologous series from 2-amino-5-phosphonovaleric acid to 2-amino-8-phosphonooctanoic acid showed depressant actions which correlated with the ability of the substances to antagonize selectively motoneuronal depolarizations induced by N-methyl-D-aspartate.

**3** 2-Amino-5-phosphonovalerate was the most potent substance of the series giving an apparent $K_D$ of 1.4 μM for the antagonism of responses to N-methyl-D-aspartate.

**4** A comparison of the (+)- and (−)-forms of 2-amino-5-phosphonovalerate indicated that the N-methyl-D-aspartate antagonist activity and the neuronal depressant action of this substance were both due mainly to the (−)-isomer.

**5** The (−)- and (+)-forms of 2-amino-4-phosphonobutyrate had different actions. The (−)-form of this substance had a relatively weak and non-selective antagonist action on depolarizations induced by N-methyl-D-aspartate, quisqualate and kainate and a similarly weak depressant effect when tested on evoked electrical activity. The (+)-form was more potent than the (−)-form in depressing electrically evoked activity but did not antagonize responses to amino acid excitants. At concentrations higher than those required to depress electrically evoked activity, the (+)-form produced depolarization. This action was blocked by 2-amino-5-phosphonovalerate.

## Introduction

The phosphonic amino acid, (±)-2-amino-5-phosphonovalerate, is a potent depressant of neuronal electrical activity in the cat and frog spinal cord, an effect that appears to be closely correlated with the ability of the substance to block excitatory amino acid receptors. In particular, this substance is a potent and highly selective antagonist of N-methyl-D-aspartate-induced responses (Davies, Francis, Jones & Watkins, 1981). Receptors sensitive to N-methyl-D-aspartate have been implicated in spinal synaptic transmission (Evans, Francis, Hunt, Oakes & Watkins, 1979; Davies & Watkins, 1979). The actions of the lower homologues, (±)-2-amino-3-phosphonopropionate and (±)-2-amino-4-phosphonobutyrate, which are the phosphono analogues of aspartate and glutamate, respectively, are less clear. Thus, these substances have been reported to have excitatory effects on neurones in the cat spinal cord (Curtis & Watkins, 1965; Watkins, Curtis & Brand, 1977), and depressant effects on electrically evoked activity in rat hippocampal preparations (White, Nadler, Hamberger, Cotman & Cummins, 1977; White, Nadler & Cotman, 1979). In the case of (±)-2-amino-4-phosphonobutyrate, depressant effects have also been observed in olfactory cortex of the rat (Hori, Auker, Braitman & Carpenter, 1980), spinal cord of the cat (Davies & Watkins, 1979) and frog (Evans *et al.*, 1979), and crayfish muscle fibres (Dudel, 1977). Such effects have been attributed to the ability of this phosphono amino acid to block excitatory amino acid-induced responses (Cull-Candy, Donnellan, James & Lunt, 1976; Davies & Watkins, 1979; Evans *et al.*, 1979). However, depression of transmission in mud-puppy retina by this substance has been ascribed to amino acid agonist rather than antagonist action, the phosphonate being proposed to mimic the action of the photoreceptor transmitter (Slaughter & Miller, 1981).

The present work compares the effects on electrically evoked and amino acid-induced responses in the frog and rat isolated spinal cord preparations of the homologous series of phosphono amino acids

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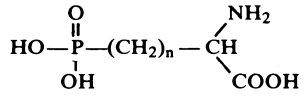
**Table 1** Structures of ω-phosphono α-carboxylic amino acids

General formula 

| *n* | *Compound* |
|---|---|
| 1 | 2-Amino-3-phosphonopropionic acid |
| 2 | 2-Amino-4-phosphonobutyric acid |
| 3 | 2-Amino-5-phosphonovaleric acid |
| 4 | 2-Amino-6-phosphonohexanoic acid |
| 5 | 2-Amino-7-phosphonoheptanoic acid |
| 6 | 2-Amino-8-phosphonooctanoic acid |

from (±)-2-amino-3-phosphonopropionic acid to the corresponding octanoic acid analogue (see Table 1). The actions of the separate (+)- and (−)-forms of the butyrate and valerate members of the series have also been studied. Antagonist actions of the phosphonates on amino acid-induced responses have been investigated using the agonists N-methyl-D-aspartate, quisqualate and kainate, which preferentially activate different types of receptors for excitatory amino acids in the mammalian and amphibian central nervous systems (Evans *et al.*, 1979; Davies & Watkins, 1979; McLennan & Lodge, 1979; reviewed by Watkins & Evans, 1981). Preliminary accounts of the actions of some of these phosphonates have been published (Evans & Watkins, 1981; Watkins, 1981).

## Methods

Most of the experiments were conducted using the isolated spinal cord of either the frog or immature rat. Some additional experiments were conducted on the isolated superior cervical ganglion of the rat. The methods of stimulation and recording, application of agonist and antagonist-containing solutions and the composition of superfusion media used for frog spinal cord, rat spinal cord and superior cervical ganglion preparations were as described by Evans & Watkins (1978) and Evans *et al.* (1979).

### *Measurement of depressant potency*

Depressant potencies were estimated from the doses of compounds which produced similar reductions in the chart-recorded amplitude of dorsal root-evoked ventral root potentials of frog spinal cord preparations. Dorsal root stimulation in frog spinal cord preparations generates a depolarization of several millivolts amplitude and several seconds' duration recorded in the corresponding ventral root (Barron & Matthews, 1938). Such ventral root potentials are believed to contain only a small monosynaptic component (Fadiga & Brookhart, 1962; see also review by Kudo, 1978). The chart recorder (Smith's Industries Servoscribe) used in the present study had a minimum time to full scale deflection of 150 ms. Consequently the peak amplitude of these electrically evoked potentials, which occurred within the initial 50 ms, was not registered.

The depression of these chart-recorded potentials by the phosphono amino acids thus represented an effect mainly on the amplitude and duration of the later components of the response. Oscilloscope recordings were made when it was desired to investigate the relative effects of substances on faster components of the response. This was particularly important in experiments on the rat spinal cord, since dorsal root-evoked ventral root potentials in this preparation have a large fast segmental component, which is probably monosynaptic (Otsuka & Konishi, 1974).

The effects of compounds on spontaneous electrical activity recorded from ventral roots were also investigated, using methods similar to those described by Allan, Evans & Johnston (1980). Such effects were observed in the presence or absence of dorsal root stimulation, and were again monitored on a chart recorder.

### *Measurement of antagonism of excitant amino acid-induced responses*

Excitant amino acid antagonist effects were measured in the presence of tetrodotoxin (0.1 μM). The procedure for producing complete blockade of transmission in the presence of tetrodotoxin 0.1 μM was as described by Evans & Watkins (1978). The agonists, N-methyl-D-aspartate, kainate or quisqualate were applied for 80 or 120 s contact time, tests for selectivity of antagonists being made using matched submaximal depolarizing responses produced by the three agonists.

Dose-ratios for the antagonism of N-methyl-D-aspartate were estimated from dose-response plots of depolarization produced by the agonist before and 30 min following application of antagonist solutions.

### *Chemicals*

The members of the ω-phosphono-α-carboxylic amino acid series from (±)-2-amino-5-phosphonovaleric acid to the corresponding octanoic acid (see Table 1) were synthesized in our laboratory by an adaptation of the method used by Chambers & Isbell (1964) for the butyric acid analogue. Racemic mixtures of the butyric and valeric acid derivatives were resolved by the method of Evans *et al.* (1979) for α-aminoadipic acid, except that L-lysine was used as the base and, after absorption of the resolved salts

on BioRad AG 50 W ($H^+$) resin, the free acids were eluted with water. The optically active acids used had the following $[\alpha]_D$ values (all determined in 6 N HCl solution): 2-amino-4-phosphonobutyric acid, +28.9° and −29.7°; 2-amino-5-phosphonovaleric acid, +23.5° and −18.4°. These rotations are close to those which would be expected from the known rotations of α-amino dicarboxylic acids (Evans *et al.*, 1979) except for the (−)-form of the valeric acid analogue, the lower rotation of which, compared with the (+)-form, indicated an 11% or higher degree of optical impurity. N-Methyl-D-aspartic acid was synthesized as described by Watkins (1962). Other chemicals were obtained from commercial sources.

## Results

### *Effect on spontaneous electrical activity of spinal cord preparations*

All the racemates of the present series produced depression of spontaneous electrical activity recorded in ventral roots of frog or rat spinal cord

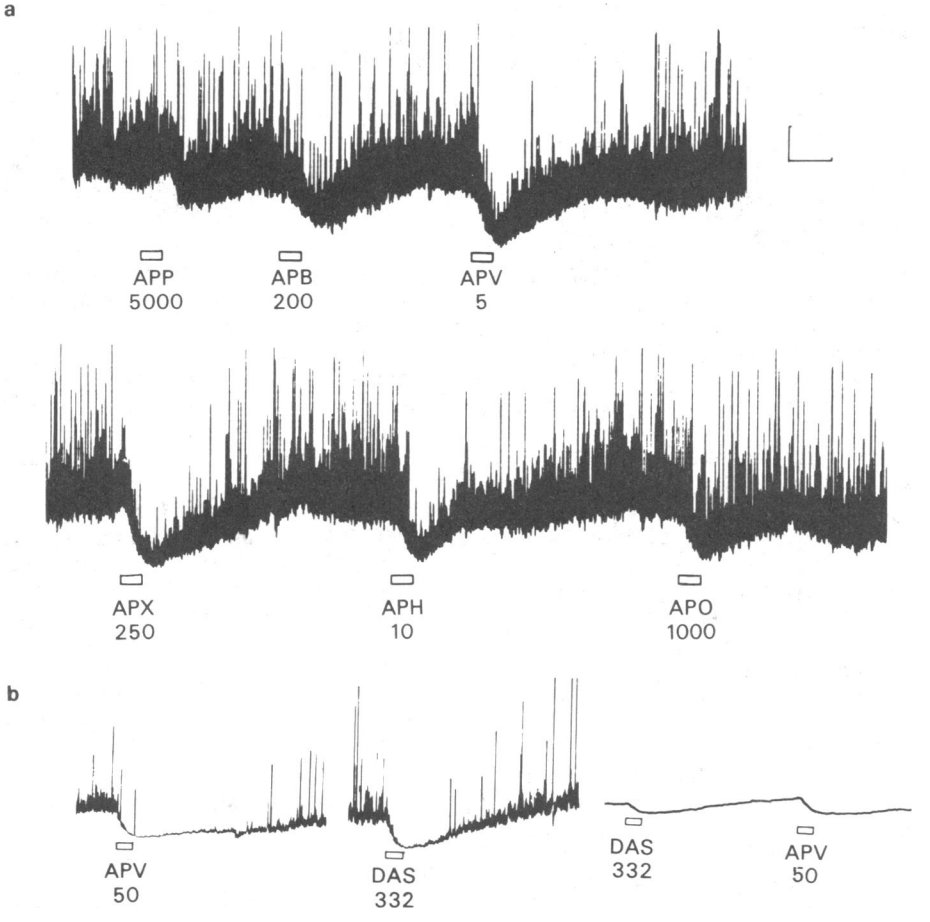



**Figure 1(a)** Effects of phosphonoamino acids on spontaneous electrical activity of a frog spinal cord. Chart recordings showing a comparison of the depressant effects of (±)-2-amino-3-phosphonopropionate (APP), (±)-2-amino-4-phosphonobutyrate (APB), (±)-2-amino-5-phosphonovalerate (APV), (±)-2-amino-6-phosphonohexanoate (APX), (±)-2-amino-7-phosphonoheptanoate (APH) and (±)-2-amino-8-phosphonooctanoate (APO) on spontaneous electrical activity recorded from a ventral root. **(b)** Depression of spontaneous electrical activity and associated hyperpolarizing action of (±)-2-amino-5-phosphonovalerate (APV) and D-α-aminosuberate (DAS) recorded from a ventral root of a frog spinal cord preparation. In the right hand trace, recorded in the presence of tetrodotoxin (0.1 μM), hyperpolarizing actions are still evident. Compounds were applied for the period indicated by the bars beneath the records at the micromolar concentrations indicated. Calibration: vertical 1 mV; horizontal, 8 min in (a), 10 min in (b).

preparations. The valerate and propionate derivatives were the most and least potent members of the series, respectively (Figure 1a). The rank order of potency for depression of spontaneous electrical activity was the same as for depression of electrically evoked activity (see below). However, based on the doses required to produce threshold effects, spontaneous electrical activity was more susceptible to the depressant action of these agents than was electrically evoked activity.

Except for the propionate and butyrate derivatives, which sometimes produced depolarization, the depressant action of the phosphonates was always accompanied by hyperpolarization recorded in ventral roots (see Figures 1 and 2). At doses of 20 $\mu$M or higher, (±)-2-amino-5-phosphonovalerate often had a significant hyperpolarizing action even after blockade of transmission with tetrodotoxin. To ascertain whether this property is unique to the phosphonates or is shared also by the carboxylate analogues, the hyperpolarizing actions of (±)-2-amino-5-phosphonovalerate and D-α-aminosuberate (Evans *et al.*, 1979) were compared. Figure 1b illustrates the results from one preparation where both compounds still produced hyperpolarization, though much reduced in magnitude, following blockade of transmission with tetrodotoxin.

*Effect on electrically evoked ventral root potentials*

Depression of dorsal root-evoked ventral root potentials by the phosphono-amino acids resembled that produced by their dicarboxylic counterparts (Evans *et al.*, 1979). Examples of the effects produced by (±)-2-amino-5-phosphonovalerate are shown in Figure 2b and d. This was the most potent substance in the series having a maximal depressant effect at doses between 20 and 50 $\mu$M (Davies *et al.*, 1981). The relative potencies of the phosphonates for such depression of dorsal root-evoked ventral root potentials in frog spinal cord preparations are represented in Figure 3a.

The initial peak (latency 4 ms) of the dorsal root-evoked ventral root potential in the frog spinal cord was resistant to (±)-2-amino-5-phosphonovalerate (Figure 2d). The depressant effect was most evident on the time to half recovery of the potential which corresponded in profile to the pen recorder peak amplitude (Figure 3b) used to compare the depressant effects of the homologues. Depression of the maximum amplitude of the dorsal root evoked ventral root potential, as measured from oscillographic traces (Figure 2d), corresponded only approximately with the pen recorder peak amplitude (Figure 3b). This is probably because the maximum amplitude (latency 7–15 ms) contained part of the initial component that was resistant to antagonism by the phosphonate.

A typical comparison of the effects of the (+)- and (−)-forms of 2-amino-5-phosphonovalerate on the amplitude of the dorsal root-evoked ventral root potential of a frog spinal cord yielded potencies of $2.00 \pm 0.16$ and $0.077 \pm 0.005$ for the (−)- and (+)-forms, respectively, relative to the racemate (mean ± s.d.; $n = 3$).

(+)-2-Amino-4-phosphonobutyrate was found to have a mixed action ranging from depressant at low concentrations (25–250 $\mu$M) to a depolarizing action at higher concentrations, whereas the (−)-form of this substance had only a depressant action. This is illustrated for the frog spinal cord in Figure 2a. The depressant action of (+)-2-amino-4-phosphonobutyrate was still evident (Figure 2b) following blockade of the depolarizing action of this substance with (±)-2-amino-5-phosphonovalerate (see below) using doses of the higher homologue that were maximally effective against electrically-evoked activity (Figure 3b).

In the rat spinal cord preparation, the effect of (+)-2-amino-4-phosphonobutyrate was specifically tested on the fast component of the dorsal root-evoked ventral root potential after depression of the slow component of this response with a mixture of (±)-2-amino-5-phosphonovalerate (50 $\mu$M) and $Mg^{2+}$ (1 mM). This mixture was used in order to suppress the activation of excitatory synaptic pathways in which N-methyl-D-aspartate receptors may be involved, the organic and inorganic antagonists presumably acting at different sites and producing multiplicative effects (Davies, Evans, Francis & Watkins, 1978; Ault, Evans, Francis, Oakes & Watkins, 1980). Figure 2c indicates that the fast component of the electrically evoked response, which was resistant to the depressant action of the (±)-2-amino-5-phosphonovalerate/$MgSO_4$ mixture, was markedly depressed by (+)-2-amino-4-phosphonobutyrate (100 $\mu$M). The threshold concentration of (+)-2-amino-4-phosphonobutyrate required to produce this effect was 5 $\mu$M, and the effect appeared to be maximal at 250 $\mu$M.

*Effect on electrically evoked ganglionic potentials*

Neither (±)-2-amino-5-phosphonovalerate (1 mM) nor (±)-2-amino-4-phosphonobutyrate (1 mM) had any significant depressant action on transmission through the isolated superior cervical ganglion of the rat.

*Depolarizing effects of phosphono amino acids*

At concentrations above 250 $\mu$M, (+)-2-amino-4-phosphonobutyrate produced depolarization recorded in ventral roots of rat and frog spinal cords (Figures 2a and b). On tetrodotoxin-blocked frog spinal cords (Biscoe, Evans, Headley, Martin & Wat-

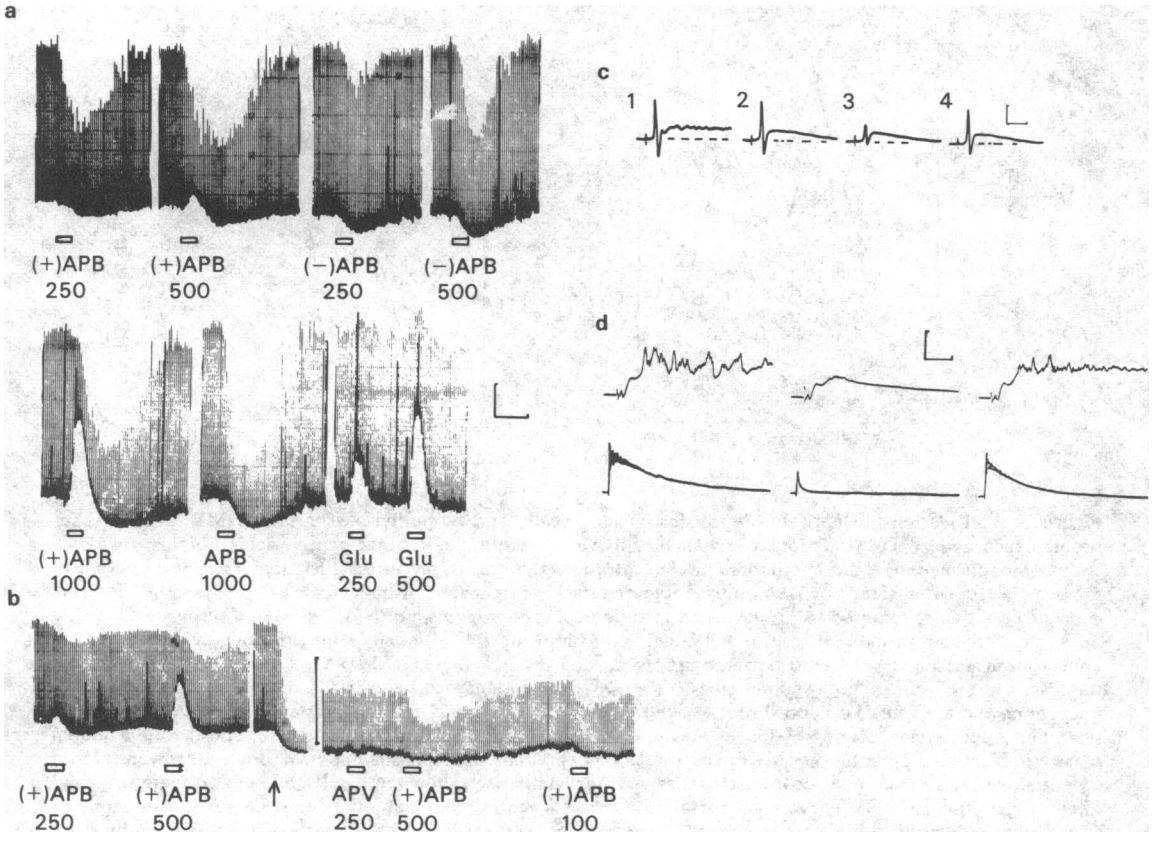



**Figure 2(a)** Comparison of the effects of (+)- and (−)-isomers of 2-amino-4-phosphonobutyrate (APB) on chart-recorded dorsal root-evoked ventral root potential (dorsal root stimulation, 2 min) of frog spinal cord preparation. Glu = L-glutamate. Numbers refer to concentrations (μM). Note the depressant effects (and associated hyperpolarizations) produced by both isomers and the additional depolarizing effect produced only by (+)APB at 500 and 1000 μM. The racemic form of the substance (APB, 1000 μM) did not cause depolarization in this preparation. The depolarizing effect of L-glutamate (500 μM), was not associated with depressant effects similar to that produced by (+)APB. Calibration 1 mV and 10 min. **(b)** Chart recording of the responses of a different frog preparation from (a), showing retained depressant effect of (+)APB following maximal depressant action of (+)-2-amino-5-phosphonovalerate (APV). Calibration as for (a). Gain increased as indicated, 20 min following introduction, at arrow, of APV (50 μM) which remained present thereafter. In this maintained presence of APV there was no further effect of additional APV (250 μM), the depolarizing effect of (+)APB was abolished, but the depressant effect of (+)APB (500 and 100 μM) was still obtained. **(c)** Oscilloscope traces showing depressant effect of (+)-2-amino-4-phosphonobutyrate ((+)APB, 100 μM) on dorsal root-evoked ventral root potential of rat spinal cord preparation. Dorsal root stimulation, 2/min. (1) Recorded 10 min before introduction of a mixture (±)-2-amino-5-phosphonovalerate (APV) (50 μM) and $MgSO_4$ (1 mM); (2) 10 min following introduction of APV/$MgSO_4$; this mixture present thereafter. Note the lack of effect of the mixture on the fast component and depression of the slower component. (3) Five min after introduction of (+)APB 100 μM, showing marked depression of the fast component. (4) Ten min following removal of (+)APB from bathing medium, showing recovery of the fast component. Calibration 4 mV, 20 ms. **(d)** Oscilloscope traces showing effect of (±)-2-amino-5-phosphonovalerate on dorsal root-evoked ventral root potentials in frog spinal cord. Vertical calibration: 5 mV. Horizontal calibration: 10 ms in upper traces, 500 ms in lower traces. The left hand records were made 10 min before and the centre records 20 min after application of (±)-2-amino-5-phosphonovalerate 200 μM. A very small presynaptic component of the potential can be seen after the stimulus artifact which is followed by the initial peak of the dorsal root-evoked ventral root potential. This initial peak is resistant to the depressant action of the phosphonate which is confined to the slower components of the response and is most evident on the lower record. Right hand records show recovery 140 min after application of phosphonate-free medium.

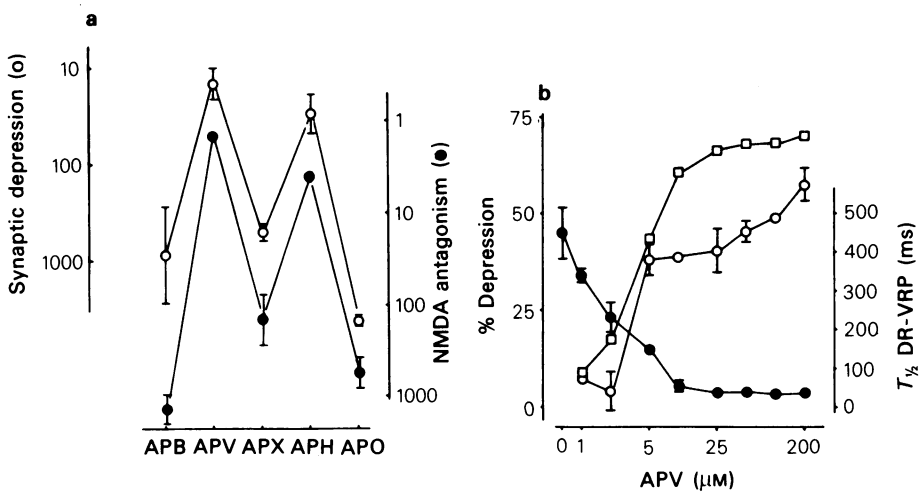



**Figure 3(a)** Relationship between depression of electrically and N-methyl-D-aspartate (NMDA)-evoked responses, recorded from ventral roots, and chain length of ω-phosphonic α-carboxylic amino acids. Left hand ordinate scale and open symbols indicate micromolar concentrations required to produce a 20% depression in amplitude of the pen-recorded dorsal root-evoked ventral root potential. The right hand ordinate scale and filled symbols indicate concentrations (μM) required to produce a dose-ratio of 2 for antagonism of NMDA-evoked responses. Vertical lines (smaller than symbols in case of APV and APH) indicate s.d. of mean, 3 preparations in each case. For abbreviations see Figure 1. A close correlation between depressant action and NMDA antagonist action is evident in the case of the four highest homologues. **(b)** Dose-dependency of depressant action of (±)-2-amino-5-phosphonovalerate (APV) on dorsal root-evoked ventral root potentials in a frog preparation. Left hand ordinate scale and open circles, % depression of maximum amplitude of the dorsal root-evoked ventral root potential measured from oscillographic records; open squares, % depression of amplitude of dorsal root-evoked ventral root potential measured from pen recorder chart, as used for comparison shown in (a). Right hand ordinate scale and filled circles, time to half recovery of dorsal root evoked ventral root potential (ms) measured from time of application of stimulus. Points are means of 3 observations; vertical lines indicate standard deviation (where not indicated, deviations are covered by the symbols).

kins, 1976), this substance had a depolarizing potency of 0.6 relative to L-glutamate; on rat spinal cord preparations it was approximately equipotent with L-glutamate. Depolarizations produced by this phosphonate, could be abolished by the addition of (±)-2-amino-5-phosphonovalerate. An example of this effect is shown in Figure 2b.

Apart from (±)-2-amino-4-phosphonobutyrate, the only other phosphonate which caused depolarization of frog ventral roots was (±)-2-amino-3-phosphonopropionate. This phosphonate had a potency of 0.03 relative to that of L-glutamate.

### *Antagonism of depolarization induced by excitant amino acids*

The present series of compounds were investigated for their ability to antagonize N-methyl-D-aspartate-, kainate- or quisqualate-induced depolarizations recorded in ventral roots of tetrodotoxin-blocked frog spinal cord preparations. The effects of the hexanoate (1 mM), heptanoate (0.1 mM) and octanoate (1 mM) derivatives were tested; N-methyl-D-aspartate-induced depolarizations were highly susceptible and kainate- or quisqualate-induced depolarizations were resistant to antagonism by these compounds (Figure 4a and Table 2).

Relative potencies for the antagonism of N-methyl-D-aspartate-induced responses were assessed in terms of the concentration of each compound required to produce a dose-ratio of 2 (Figure 3a). A typical dose-response curve displacement is illustrated in Figure 4b. A correlation is apparent between the relative potencies of the substances as antagonists of N-methyl-D-aspartate-induced responses and their potencies as depressants of electrically evoked activity (Figure 3a). (±)-2-Amino-3-phosphonopropionate was not tested for N-methyl-D-aspartate antagonism. the high antagonist potency of (±)-2-amino-5-phosphonovalerate against N-methyl-D-aspartate-induced responses enabled dose-ratios of several hundred to be obtained with this compound and thus allowed the construction of a Gaddum-Schild plot over a wide range of concentration. Figure 4c shows such a plot of pooled results from 10 frog preparations; a linear relationship with

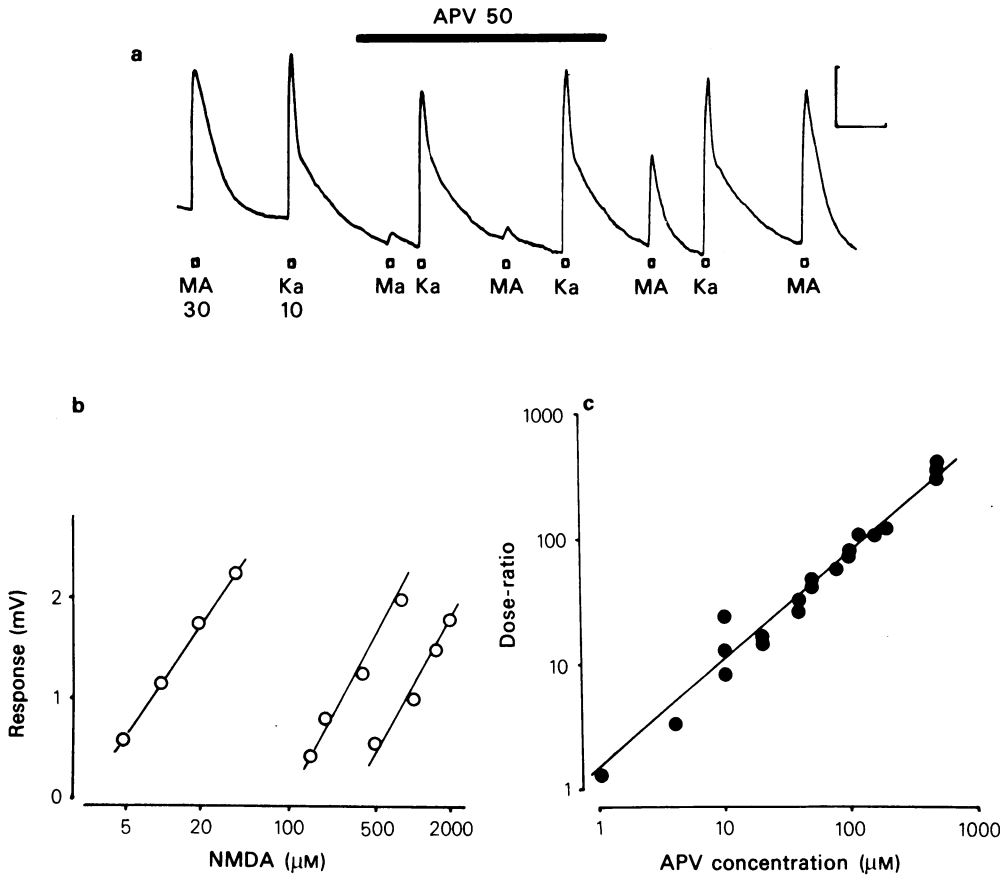



**Figure 4(a)** Effect of (±)-2-amino-5-phosphonovalerate (APV), applied for period indicated, on depolarizations evoked by kainate (Ka) or N-methyl-D-aspartate (MA) and recorded in a ventral root of a tetrodotoxin-blocked frog spinal cord preparation. Calibration: 1 mV, 10 min. Numbers refer to concentrations, (μM), Note the selective antagonism of the MA-induced responses by APV. **(b)** Displacement by (±)-2-amino-5-phosphonovalerate (APV) of dose-response plots for N-methyl-D-aspartate (NMDA)-evoked responses measured in a ventral root of a frog spinal cord preparation. Left hand plot, in the absence of antagonist; centre and right hand plots, in the presence of 40 and 120 μM APV, respectively. The lines were fitted by the method of least squares and the slopes are not significantly different from each other (Student's $t$ test; $P > 0.1$). **(c)** Gaddum-Schild plot of pooled data from 10 frog preparations. Depolarizing responses were evoked by 2 min applications of N-methyl-D-aspartate before and 25 min following application of (±)-2-amino-5-phosphonovalerate (APV). Tetrodotoxin (0.1 μM) was present throughout. The line was fitted by the method of least squares and yields an apparent $K_D$ (mean ± s.e.) of 1.43 μM ± 0.01. The slope is not significantly different from unity ($P > 0.1$).

a slope of unity was obtained. From this relationship an apparent $K_D$ of 1.4 ± 0.01 (mean ± s.e.mean, $n = 10$) was calculated for antagonism of N-methyl-D-aspartate-induced responses by (±)-2-amino-5-phosphonovalerate.

It was of interest to determine if (±)-2-amino-5-phosphonovalerate produced its antagonism at the same site as other structurally related antagonists. Therefore, dose-ratios for antagonism produced by mixtures of this phosphonate and (±)-α, ε-diaminopimelic acid were measured. Pooled data from two frog spinal cord preparations yielded a dose-ratio of 17.6 ± 1.5 (mean ± s.d.; $n = 8$) for antagonism of N-methyl-D-aspartate-induced depolarizations by a mixture of (±)-2-amino-5-phosphonovalerate (10 μM) and α, ε-diaminopimelic acid (1 mM).

Both (−)- and (+)-2-amino-5-phosphonovalerate depressed N-methyl-D-aspartate-induced ventral root depolarization. Based on the concentrations necessary to depress such N-methyl-D-aspartate-induced responses to the same extent, the ratio of the

**Table 2** Antagonism of excitatory amino acid-induced depolarization frog spinal cord by ω-phosphono α-carboxylic amino acids

| Antagonist | *Size of response to agonist (% of control) in the presence of antagonist* NMDA | Quisqualate | Kainate |
|---|---|---|---|
| (±)APV* (50 μM) | 5±2 (4) | 95± 2 (3) | 97± 2 (6) |
| (±)APX (1000 μM) | 7±4 (4) | 100± 3 (4) | 94±11 (4) |
| (±)APH (100 μM) | 4±4 (4) | 99±13 (4) | 98± 6 (4) |
| (±)APO (1000 μM | 58±4 (4) | 113±11 (4) | 92± 2 (4) |

The values given are the magnitudes of the depolarizations measured in tetrodotoxin-containing medium in the presence of an antagonist expressed as a percentage (mean ± s.e.mean) of the appropriate control responses measured in the absence of the antagonist. Control responses (approximately 2 mV) were matched prior to introduction of antagonist. The number of experiments is given in parentheses *Values from Davies *et al.*, 1981. Abbreviations: APV = (±)-2-amino-5-phosphonovalerate, APX = (±)-2-amino-6-phosphonohexanoate, APH = (±)-2-amino-7-phosphonoheptanoate, APO = (±)-2-amino-8-phosphonooctanoate.

potency of the (−)-relative to the (+)-form was 31 ± 2.5 (mean ± s.d.; $n = 3$).

The (−)- and (+)-forms of 2-amino-4-phosphonobutyrate had different effects. The (−)-form (0.5 mM) antagonized matched responses (approximately 2 mV depolarization) produced by N-methyl-D-aspartate, kainate and quisqualate by 50 ± 2, 46 ± 12, and 12 ± 2% (mean ± s.d., $n = 3$) respectively. Based on dose-ratios, its effectiveness in depressing N-methyl-D-aspartate-induced responses was between two and three orders of magnitude weaker than (−)-2-amino-5-phosphonovalerate (potency ratio 373 ± 41, $n = 3$). (−)-2-Amino-4-phosphonobutyrate also antagonized depolarizations produced by the (+)-form of the substance.

The relatively potent depressant action of (+)-2-amino-4-phosphonobutyrate on evoked electrical activity (Figure 2a, b and c) suggested that this compound also might have amino acid antagonist properties. However, no blocking action on responses to N-methyl-D-aspartate, kainate or quisqualate were observed at concentrations of this phosphonate up to 250 μM, at which concentration maximal depression of electrically evoked activity was observed. At higher concentrations measurement of the depressant effects of (+)-2-amino-4-phosphonobutyrate was complicated by its direct depolarizing action. To eliminate this depolarizing action, which is susceptible to blockade by (±)-2-amino-5-phosphonovalerate (Figure 2b), further tests for the ability of (+)-2-amino-4-phosphonobutyrate to antagonize kainate- or quisqualate-induced responses were conducted on preparations treated with (±)-2-amino-5-phosphonovalerate (50 μM), or a mixture of this substance (50 μM) and $MgSO_4$ (1 mM), to block N-methyl-D-aspartate receptors. Under these conditions, (+)-2-amino-4-phosphonobutyrate (1 mM) again had no action on kainate- or quisqualate-induced responses.

## Discussion

Current evidence supports the existence of three major types of excitant amino acid receptor activated selectively by the agonists N-methyl-D-aspartate, kainate and quisqualate in the amphibian and mammalian central nervous system (see Watkins & Evans, 1981). A previous structure-activity investigation of straight chain α-amino dicarboxylic acids in isolated spinal cord preparations has revealed that the D-isomers of homologues higher than glutamate have N-methyl-D-aspartate antagonist activity, which is correlated with their central depressant potencies (Evans *et al.*, 1979). The selective N-methyl-D-aspartate antagonist activity of these compounds is characterized by the resistance to antagonism of depolarization evoked by kainate or quisqualate. The hexanoate, heptanoate and octanoate members of the present series also showed this profile of selectivity which has been reported previously for the valerate derivative (Davies *et al.*, 1981).

The profile of depressant action against chain length for the present series is remarkably similar to the corresponding structure-activity profile found for monoamino dicarboxylic acids, the most notable feature being the marked reduction in activity at (±)-2-amino-6-phosphonohexanoate, corresponding to α-aminopimelate in the dicarboxylate series (Evans *et al.*, 1979). The loss in activity associated with this chain length (5 carbon atoms between the acidic groups, see Table 1), may reflect critical structural features of N-methyl-D-aspartate receptor sites and mode(s) of antagonist binding. Peaks of activity corresponding to (±)-2-amino-5-phosphonovalerate and (+)-2-amino-7-phosphonoheptanoate were apparent on either side of this least effective chain length, as also observed in the dicarboxylate series.

For compounds containing 4–6 carbon atoms in the interconnecting chain (valerate to octanoate derivatives) the parallelism between synaptic depressant potency and N-methyl-D-aspartate antagonist activity is even more striking in the present study (Figure 3a) than previously observed in the corres-

ponding comparison within the dicarboxylate series (Evans *et al.*, 1979). This is probably due to a difference in the methods used to compare potencies for the two actions. The present comparison was based on a null-point method in which concentrations required to produce similar effects were estimated. In the dicarboxylate study (Evans *et al.*, 1979) different effects produced by the same concentrations of each compound were compared.

The similarity in structure-activity profile between the ω-carboxylate and ω-phosphonate series suggests that both types of compound act at the same site. This suggestion is supported by the additive antagonism produced by a mixture of (±)-α, ε-diaminopimelic acid and (±)-2-amino-5-phosphonovalerate. The dose-ratio reported previously for 1 mM (±)-α-ε-diaminopimelic acid alone was 9.3 ± 0.4 (mean ± s.e.mean; $n = 4$) (Davies *et al.*, 1978), while the corresponding value for the antagonism produced by 10 μM (±)-2-amino-5-phosphonovalerate, calculated in the present work from the data of Figure 4c, is 8.0. The sum of these dose-ratios is very similar to the observed value produced by the mixture of these antagonists.

The linear Gaddum-Schild plot for antagonism of N-methyl-D-aspartate-induced depolarizations by (±)-2-amino-5-phosphonovalerate suggests competition between agonist and antagonist, the slope of unity indicating a one to one molecular interaction between antagonist and receptor. The depression of electrically evoked activity produced by 2-amino-5-phosphonovalerate may result from a similar competitive antagonism of an excitatory amino acid transmitter. Another criterion of competitive antagonism is the maintenance of maximum response amplitude in the presence of antagonist. However, such tests cannot be applied with excitatory amino acids, since application of sufficiently high concentrations of such agonists to produce maximal responses in spinal cord preparations results in responses which decline rapidly in amplitude (see Evans, 1980), a phenomenon that is probably related to the neurotoxic action of the amino acid excitants (see Olney, 1978).

Local anaesthetic substances including procaine (Evans, 1978) and tetrodotoxin (R.H. Evans, unpublished observations) produce a long lasting hyperpolarization recorded in ventral roots of isolated spinal cord preparations suggesting that spontaneous action potentials may result in the tonic release of a depolarizing transmitter onto motoneurones in these preparations. Since, in the present study, (±)-2-amino-5-phosphonovalerate had no effect on ganglionic transmission, it may be concluded that this compound, in common with other N-methyl-D-aspartate antagonists (Evans & Watkins, 1978), is devoid of local anaesthetic activity. Thus, the hyperpolarization which accompanies the depressant effect of these antagonists may be caused by the blockade of a spontaneously released transmitter which acts at N-methyl-D-aspartate receptors. However, the partial resistance to tetrodotoxin of this hyperpolarizing effect, observed in some experiments, suggests either that part of this spontaneous transmitter release occurs independently of conducted activity, that the blockade of conduction within the preparation was incomplete (despite loss of the dorsal root-evoked ventral root potential), or, most importantly, that the antagonists have direct membrane hyperpolarizing actions which are independent of competition for N-methyl-D-aspartate receptor sites. The last possibility is unlikely however, since a direct hyperpolarizing effect at sites other than N-methyl-D-aspartate receptors would not be expected to result in a selective depression of N-methyl-D-aspartate-induced responses. Moreover, it should be noted that the N-methyl-D-aspartate antagonist, D-α-aminoadipate, which produces ventral root hyperpolarizations similarly to (±)-2-amino-5-phosphonovalerate, antagonizes amino acid-induced depolarizations in cultured neurones from mouse spinal cord but does not hyperpolarize these cells (Bergey, Martin & Hermes, 1980). Similarly, another N-methyl-D-aspartate antagonist, γ-D-glutamylglycine (Francis, Jones & Watkins, 1980), which also causes ventral root hyperpolarization (R.H. Evans, A.A. Francis and J.C. Watkins, unpublished observations) antagonizes the amino acid-induced depolarization of CA1 hippocampal neurones in the absence of effect on the neuronal membrane potential or input resistance (Segal, 1981).

The stereoselective N-methyl-D-aspartate antagonist actions observed with the (−)-forms of both 2-amino-4-phosphonobutyrate and 2-amino-5-phosphonovalerate suggest that the stereoselectivity of the phosphonates is the same as that observed for the dicarboxylate series (Evans et al., 1979), the N-methyl-D-aspartate antagonist activity residing in the D(−)-isomers. As predicted in the earlier study with the racemic mixture (Davies *et al.*, 1981) and confirmed in the present study, (−)-2-amino-5-phosphonovalerate is the most potent N-methyl-D-aspartate antagonist discovered to date. While the (+)-form of the substance also showed activity as an antagonist of N-methyl-D-aspartate-induced responses and as a depressant of electrically evoked activity, such actions could have been due to slight optical impurity of the (+)-form, the relative potencies of the (+)- and (−)-forms suggesting a possible 3–4% contamination of the (+)- with the (−)-form.

The properties of (+)- and (−)-2-amino-4-phosphonobutyrate represent a divergence from those of the other members of this phosphonate series. The relatively non-selective and weak an-

tagonism of N-methyl-D-aspartate, kainate or quisqualate responses produced by the (−)-form of this substance was similar in character to the effects of the racemate reported previously (Evans *et al.*, 1979) and such an action on excitatory amino acid transmitter receptors could contribute to the depressant action of this isomer on dorsal root evoked ventral root potentials. However, the depressant action of the (+)-isomer on evoked electrical activity, which was more potent than that of the (−)-isomer, and which has been observed also in hippocampal slice preparations (Koerner & Cotman, 1981), is unlikely to be due to blockade of excitatory amino acid transmitter receptors since responses to excitant amino acids were not antagonized by this substance. This depressant action of (+)-2-amino-4-phosphonobutyrate was still observed in the presence of maximal depressant levels of (±)-2-amino-5-phosphonovalerate, confirming a fundamental difference between the depressant mechanisms of these two agents. Such a difference is also indicated by the selective depression of the slow component of the dorsal root evoked ventral root potential in the rat spinal cord preparation by the latter substance, whereas the former had a potent depressant effect on the fast component.

The depolarizing action of (+)-2-amino-4-phosphonobutyrate, seen only at concentrations exceeding those necessary for depression of electrically evoked activity, was highly susceptible to antagonism by (±)-2-amino-5-phosphonovalerate, indicating that this depolarizing action is mediated mainly by N-methyl-D-aspartate receptors. In this respect (+)-2-amino-4-phosphonobutyrate differs from its structural analogue, L-glutamate, which seems to produce its excitatory effects mainly at other excitatory amino acid receptors (Davies & Watkins, 1979; Evans *et al.*, 1979; McLennan & Lodge, 1979; Ault *et al.*, 1980). Previous inconsistencies in the reported actions of (±)-2-amino-4-phosphonobutyrate might be explained by these different properties of the separate isomers, combined with different dose-response profiles for these effects.

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