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Research report

The anti-epileptic effect of 3-aminopropylarsonate on electrically-kindled and *N*-methyl-D-aspartate-kindled amygdala

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Abstract

The effects of 3-aminopropylarsonate, an arsono analogue of GABA, was tested on the development of electrically-kindled amygdala and on the expression of generalized seizure activity in electrically and NMDA fully amygdala-kindled rats. Intra-amygdaloid microinjection of 3-aminopropylarsonate (10 nmol in 0.5 μl injection vehicle) inhibited electrical epileptogenesis by keeping the seizure score at or below stage 1 on the Racine scale, and the afterdischarge duration (ADD) at or below 19.70 ± 4.59 s. The effect was reversible after withdrawal of the drug, since the animals developed a generalized seizure activity when kindling stimuli continued in the absence of drug. In fully electrically kindled animals with stage 5 amygdala-kindled seizures, the drug increased afterdischarge threshold (ADT) by 30–70%, without any effect on mean seizure score or ADD. The changes were reversible after 7 days. In fully NMDA-kindled rats, intra-amygdala administration of 3-aminopropylarsonate (10 nmol/0.5 μl) 20 min before injection of NMDA (4 nmol/0.5 μl) reduced the seizure score from $3.80 \pm 0.37(5)$ on the Racine scale to $0.83 \pm 0.40(6)$ ($P < 0.01$). The effect was partially reversible after washing with phosphate buffer. 2-Amino-4-arsonobutyrate, the analogue of glutamate, had no effect on seizure score following treatment with the same concentration of the drug and the same route of injection. The inhibitory effect of 3-aminopropylarsonate on NMDA kindled activity was dose-dependent, since higher doses of NMDA reduced the effect of the drug. The effect of 3-aminopropylarsonate was also selective to NMDA receptors since it had no effect on kainate-induced seizures. With both models of kindling, no gross behavioural abnormalities were observed 3–6 months after treatment with the drug. These findings show the potent antiepileptogenic and anti-convulsant activity of the arsonoanalogue of GABA which appears to be non-toxic and therefore potentially useful as the basis for developing a new family of clinically useful anticonvulsants for treating epilepsy.

Keywords: 3-Aminopropylarsonate; 2-Amino-4-arsonobutyrate; Epileptogenesis; Electrical kindling; *N*-Methyl-D-aspartate; Seizure; γ-Aminobutyric acid; Kainate; Amygdala

1. Introduction

Pharmacological studies have revealed that the excitatory amino acids (EAA) glutamate and aspartate, and the inhibitory amino acid GABA are centrally involved in the basic mechanisms generating epileptic seizures, and in epileptogenesis [2–5]. Antagonists, particularly those specifically acting on the *N*-methyl-D-aspartate (NMDA) receptor type, e.g. 2-amino-5-phosphonovaleric acid (AP5) and 2-amino-7-phosphonoheptanoic acid (AP7), have been found to inhibit seizures in both rodent and primate models of epilepsy [5–8,24]. Moreover, these antagonists have been shown to inhibit the development of electrically kindled epilepsy [3,5,6,9,10,12,25,28,34,35] and chemically induced seizures following repeated injections with NMDA [3,13,26,27] or repeated microinjections of glutamate or aspartate [11,26]. The presynaptic metabotropic glutamate receptor agonist α-amino-4-phosphonobutyrate (L-AP4) blocked excitatory transmission in the hippocampus [17] and blocked cortical spiking and peripheral myoclonic jerking in cobalt-induced epileptic animals [7]. 3-Aminopropylphosphonate the phosphono-analogue of GABA has a strong depressive action on central nervous systems with partial $GABA_B$-receptor agonist activity [18,19,22].

The need to look for more potent anticonvulsants in-

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cluding new analogues of GABA, has lead to the synthesis of arsono-analogues which are more potent and more stable than the phosphono analogues [15]. 3-Aminopropylarsonate, an analogue of GABA, was synthesised by Ali and Dixon in 1993 [1].

In the present study the effects of 3-aminopropylarsonate (3-APA) on the development of electrical kindling and on seizure-suppression in fully electrically and NMDA-kindled animals was investigated.

2. Materials and methods

2.1. Animals and surgery

Experiments were performed on male Sprague-Dawley rats weighing 280–320 g, which were anaesthetised with a halothane/nitrous oxide mixture (2–3% halothane in 2:1 mixture of oxygen and nitrous oxide). During light anaesthesia a combined guide-cannula and stainless steel bipolar electrode unit was implanted into the right basolateral amygdala using the following co-ordinates for the tip of the bipolar electrodes: AP = −0.8, L = −3.8, V = −8.8, from the skull surface. The combined unit was fixed to the skull with the aid of two stainless steel anchor screws, using cyanoacrylate cement and zinc powder. Details are as described elsewhere [10].

Animals were kept in separate cages with free access to food and water and were left for two weeks to recover from surgery before starting chemical or electrical kindling.

2.2. Electrical kindling

Electrical kindling was performed by giving a daily electrical stimulus (which was 125% of the threshold current) to the amygdala. The afterdischarge threshold was determined using the method of ascending limits [10] and the progressive daily development of seizure activity was rated on a 5-point scale based on that of Racine [29]: Stage 1, facial myoclonus and vibrissae twitching; Stage 2, jaw myoclonus and head bobbing; Stage 3, unilateral forelimb myoclonus; Stage 4, bilateral forelimb myoclonus; and Stage 5, bilateral myoclonus with repeated rearing and falling.

Afterdischarge duration (ADD) was calculated from the EEG, and electrical responses were recorded on a Grass Model 79D polygraph. The generalized seizure threshold (GSTs) were determined following three consecutive fully kindled (stage 5) seizures. The action of the drugs on the development of electrical kindling was tested by injecting the drug (10 nmol/0.5 μl), or vehicle alone (0.5 μl) intracerebrally, 20 min before applying each kindling stimulus over the whole period. After 8 days the drug was withdrawn from the experimental animals and the kindling process was continued in the absence of the drug. All experiments were performed on awake unrestrained animals which displayed normal behaviour as judged by food and water intake and general motor and exploratory behaviour.

2.3. NMDA kindling

For chemical kindling with *N*-methyl-D-aspartate, this compound was focally micro-injected on a daily basis into the basolateral amygdala at a dose of 4 nmol in a total volume of 0.5 μl of 50 mM phosphate buffer, pH 7.4 [13]. The treatment continued until the development of fully kindled seizures, at a score-level of 4 or 5 on the Racine scale. Animals were observed for behavioural changes for 30 min. post-injection. Motor seizure activity was rated on a scale of 1 to 5 based on that of Racine (see above).

In animals which were fully kindled by repeated daily NMDA injections, the anti-convulsive activity of 3-aminopropylarsonate (10 nmol/0.5 μl) was tested. It was injected 5 minutes before the injection of different concentrations of NMDA (4 to 40 nmol) in 0.5 μl buffer, or of kainate (4 nmol in 0.5 μl).

During all experiments the severity of the accompanying motor seizures was compared between 3-aminopropylarsonate treated and phosphate-buffer pretreated animals.

2.4. Drugs

The arsono compounds were kind gifts from Dr. H.B.F. Dixon and Dr. B.R.S. Ali from the Biochemistry Department of Cambridge University. NMDA and Kainate were purchased from Tocris Cookson (Bristol, UK).

3. Results

3.1. Action of 3-aminopropylarsonate on electrically-induced epileptogenesis

In these experiments the mean afterdischarge threshold (ADT) measured was 348 ± 50 μA (mean ± S.E.M.; n = 10) with an initial seizure score of 1.60 ± 0.31 and a mean afterdischarge duration (ADD) of 19.70 ± 4.59 s. The estimated daily stimulus current was 435 ± 62 μA.

Repeated daily stimulations 125% of the ADT of each animal, caused a gradual increase of ADD and in the severity of the motor seizure responses as shown in mean seizure score (Fig. 1). Control animals reached stage 5 seizure activity after 6 days and were classified as fully kindled after three consecutive stage 5 seizures. After 8 days the seizure score was 4.50 ± 0.29 and the mean afterdischarge duration was 89.75 ± 13.16 s.

Treated animals were injected (into the amygdala) with 3-aminopropylarsonate (10 nmol/0.5 μl) 20 min before

applying the 1-s kindling stimulus. This drug inhibited the development of the kindled epileptic state. Thus, kindling did not proceed on average beyond Racine stage 1 seizure activity, with an ADD of 6.20 ± 2.63 s (mean $\pm$ S.E.M.; $n = 5$). After withdrawal of the drug treatment, the kindling procedure was continued, with only vehicle phosphate buffer being microinjected instead of 3-aminopropylarsonate. The seizure score and ADD subsequently started to increase gradually toward generalized seizure activity, reaching the values of 3.67 ± 0.88 in mean seizure score, and 72.50 ± 19.34 s in ADD, 6 days after withdrawal of the drug (Fig. 1).

3.2. Action of 3-aminopropylarsonate on electrically fully kindled seizures

The anti-epileptic activity of the arsono compound was also tested on fully electrically-kindled animals with seizure scores 4.20 ± 0.58 ($n = 5$). Fig. 2 shows that the first injection with 3-APA (10 nmol/0.5 μl) increased the after discharge threshold (ADT) by 31% ($P < 0.01$). The second day's injection increased this to 60% ($P < 0.01$) and the third daily injection raised it to 74% ($P < 0.02$). The ADT diminished to a 34% increase after 24 h, and the increase was completely reversed after 6 days with some

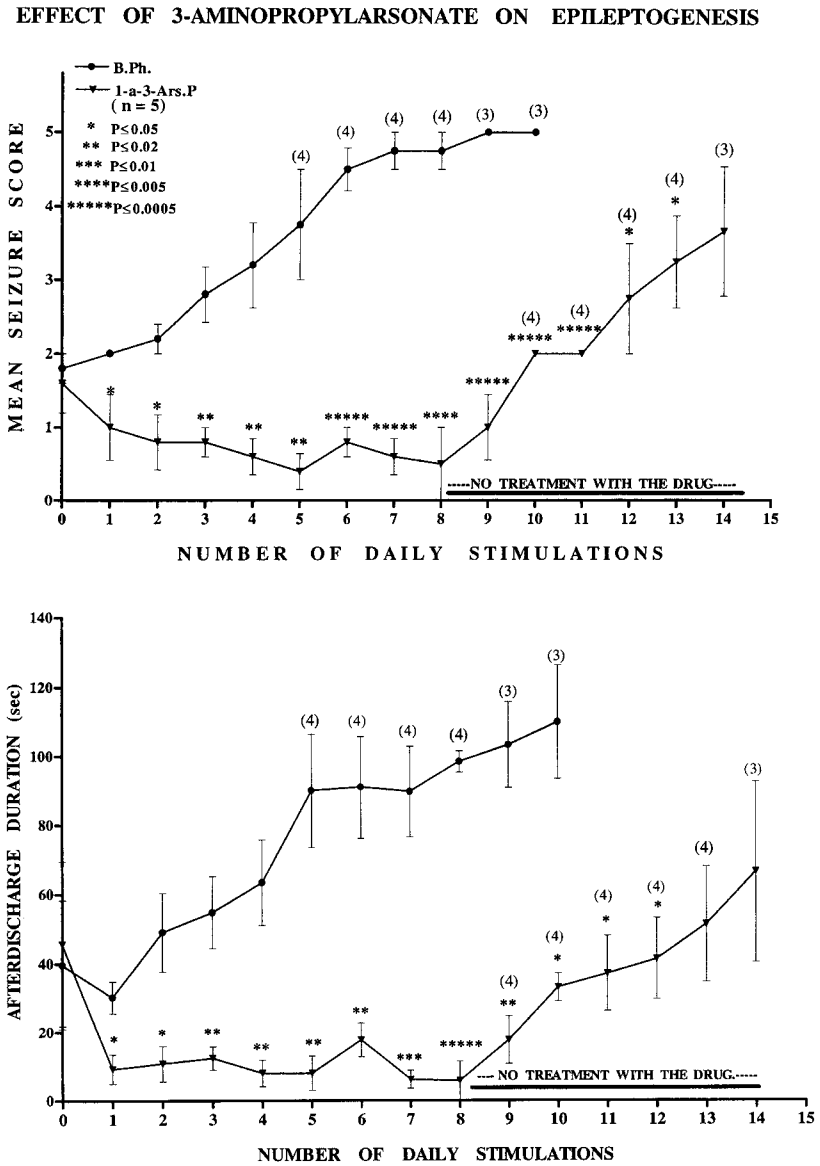

Fig. 1. The development of electrical kindling and its inhibition by 3-aminopropylarsonate. Values shown are mean seizure responses and afterdischarge durations, following repeated daily stimulation. Values are mean $\pm$ S.E.M.; $n = 5$ except where indicated in parentheses. Statistical significance was assessed using Student's t-test. Control animals received buffer phosphate (B. Ph) 0.5 μl 20 min before stimulus, and treated animals received an intra-amygdaloid injection of 3-aminopropylarsonate (1-a-3-ArsP) 10 nmol, 0.5 μl. Following 8 daily injections the drug was discontinued and was replaced with phosphate buffer.

tailing-off variation over 7–11 days of buffer injection (Fig. 2). These changes in ADT were not accompanied by any significant change in mean seizure score or afterdischarge duration.

3.3. NMDA kindling

Repeated daily focal intra-amygdaloid injection of NMDA (4 nmol) in 0.5 μl vehicle evoked seizure of progressively increasing severity from facial myoclonus to head bobbing, jaw myoclonus, unilateral forelimb jerking, to rearing and falling backwards with bilateral myoclonus. The animals developed a generalized seizure activity at the level of stages 4 or 5 on the Racine scale following a mean (±S.E.M.) of 16.00 ± 1.44 ($n = 6$) NMDA injections (mean seizure score 4.17 ± 0.40 ($n = 6$)). In one animal, stage 3 activity was achieved, but this did not progress to stage 4 activity (Table 1). One month after NMDA-induced kindling, a further single dose of NMDA (4 nmol) evoked seizure responses at the Racine score-level of 3.80 ± 0.37 (mean ± S.E.M.; $n = 5$).

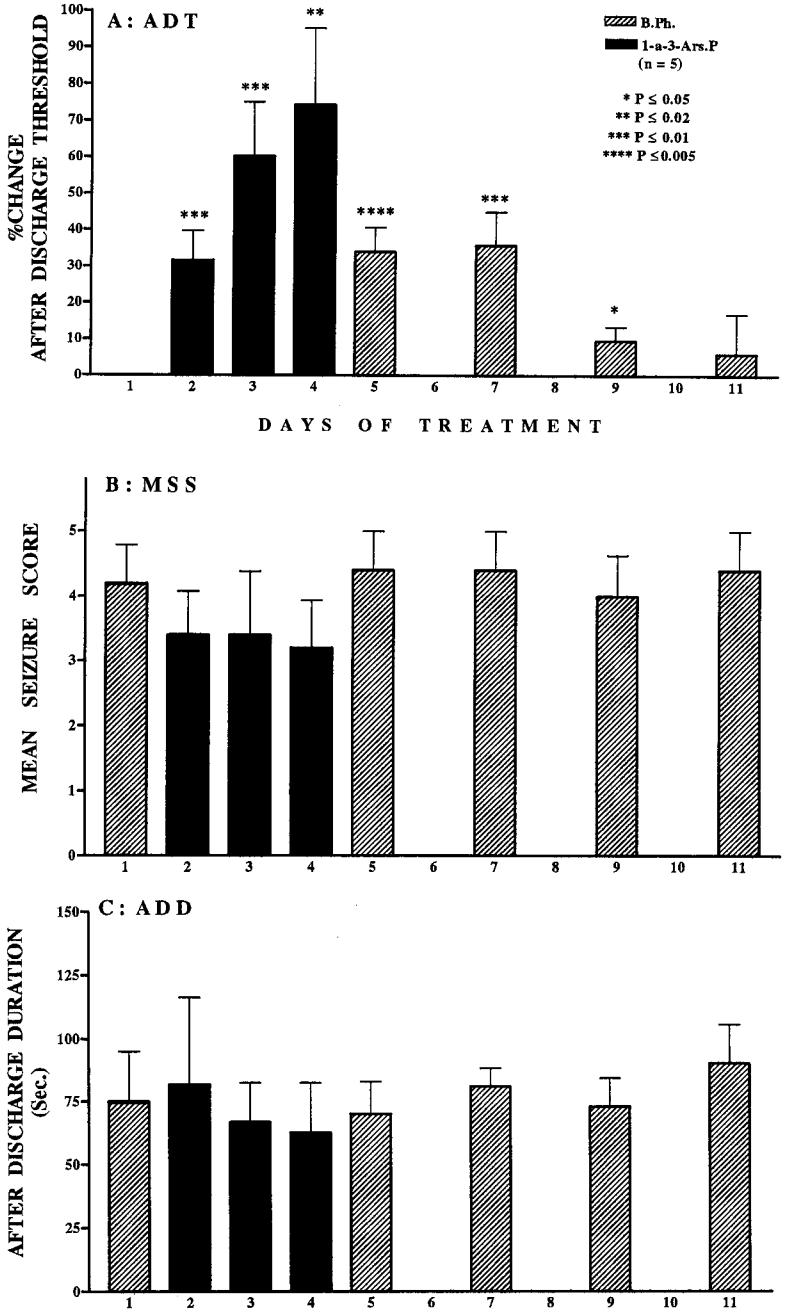

Fig. 2. The influence of 3-aminopropylarsonate on the parameters of fully electrically kindled amygdala seizures (percentage change in after-discharge threshold (ADT), mean seizure score (MSS) and afterdischarge duration (ADD)). Values shown are mean ± S.E.M.; $n = 5$. B.Ph, phosphate buffer; 1-a-3 ArsP, 3-aminopropylarsonate.

Table 1
The maximal seizure score and the number of injections to MSS following repeated focal NMDA administration

Animal	Maximal seizure score	No. of injections to maximal seizure score
N1	4	18
N2	5	15
N3	4	14
N4	5	22
N5	3	15
N6	5	21
Mean ± S.E.M.	4.17 ± 0.40 (6)	16.00 ± 1.44 (6)

Animals (N1–N6) were given daily focal intra-amygdaloid microinjection of NMDA 4 nmol in 0.5 μl phosphate buffer. The maximal seizure score reached the number of injections to maximal seizure stage is shown for each animal.

3.4. Action of 3-aminopropylarsonate on NMDA kindled seizures

Table 2 shows that pretreatment of NMDA kindled animals with 3-aminopropylarsonate (10 nmol/0.5 μl vehicle) 5 min before injection of NMDA (4 nmol) reduced the mean seizure score by 78% ($P < 0.01$) from 3.80 ± 0.37 to 0.83 ± 0.40 (mean ± S.E.M.; $n = 6$). At 1 to 7 days after injection of the 3-APA, injection of phosphate buffer before NMDA, reduced this to a seizure level of 2.50 ± S.E.M.; $n = 14$), but it was still significantly lower (by 34% ($P < 0.05$) than control values.

Table 2
Influence of 3-aminopropylarsonate and 2-amino-4-arsonobutyrate on seizure score of NMDA kindled animals

Pre-treatment	Treatment	Seizure score
A:		
Buffer phosphate	NMDA (4 nmol)	3.80 ± 0.37 (5)
3-a-Propyl arsonate (1–3 days)	NMDA (4 nmol)	0.83 ± 0.40 (6) * *
Buffer phosphate (1–7 days after 3-a-P. arsonate)	NMDA (4 nmol)	2.50 ± 0.17 (14) *
B:		
Buffer phosphate	NMDA (4 nmol)	3.80 ± 0.37 (5)
2-a-4-Arsono butyrate (1–3 days)	NMDA (4 nmol)	3.77 ± 0.40 (9)
3-a-Propyl arsonate (1–3 days after 2-a-4-Ars. But.)	NMDA (4 nmol)	1.56 ± 0.29 (9) *
Buffer phosphate (1–4 days after 3-a-P. arsonate)	NMDA (4 nmol)	2.67 ± 0.14 (12) *

All animals were fully kindled with NMDA (4 nmol in 0.5 μl vehicle). The effect of the arsono analogues was tested following a single focal micro-injection of NMDA. Control animals were treated with the same volume of buffer phosphate 5 min before injecting NMDA (4 nmol). Values are mean ± S.E.M. for the number of experiments indicated in brackets. A total of 6 NMDA kindled animals was used for these experiments, with multiple injections as indicated by 'n'. These six animals are identified (maximal seizure score) in Table 1. The periods indicated above (e.g. 1–3; 1–7) are the time ranges in which the repeated injections were made.
Block A shows 3-APA treatment, with control phosphate buffer injections before and after this treatment. Block B shows treatment with 2-a-4-arsonobutyrate, followed by 3-APA, with control phosphate buffer injections before and afterwards.
Significance of differences between control and treated were assessed using Student's *t*-test. * $P \leq 0.05$; * * $P \leq 0.01$.

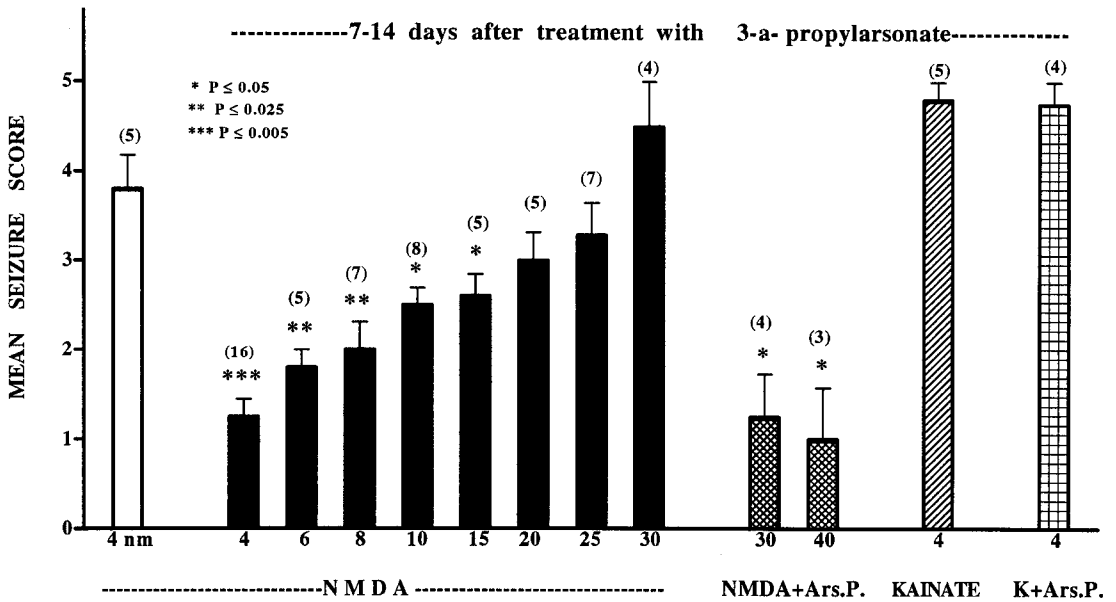

Fig. 3. Influence of 3-aminopropylarsonate on mean seizure score of fully NMDA-kindled animals. Animals were focally pretreated with 3-aminopropylarsonate (10 nmol in 0.5 μl) 5 min prior to injection of different doses of NMDA (4–40 nmol) or kainate (4 nmol). Control animals were injected with the same volume of phosphate buffer 5 min prior to injection of NMDA or kainate. Values shown are mean seizure responses (± S.E.M.) for the number of experiments indicated in parentheses. Significance of differences between 3-aminopropylarsonate-treated and buffer-treated controls were assessed using Student's *t*-test. K + Ars.P. = kainate plus 3-a-propylarsonate.

Similar treatment with 2-amino-4 arsonobutyrate (the arsono analogue of glutamate) had no effect on the mean seizure score of 3.77 ± 0.40 ($n = 9$). When the same animals were treated with 3-aminopropylarsonate (10 nmol), 1 to 3 days after treatment, with arsonobutyrate, it reduced the evoked seizure responses induced by NMDA by 59% ($P < 0.05$), i.e. from 3.80 ± 0.37 to 1.56 ± 0.29 (mean $\pm$ S.E.M.; $n = 9$). Further treatment with phosphate buffer (0.5 μl) 5 min before NMDA, significantly reduced the effect to a Racine seizure score of 2.67 ± 0.14(12) ($P < 0.05$) (Table 2).

Fig. 3 shows that 7–14 days after treatment with 3-aminopropylarsonate, this compound was still effective in reducing the evoked seizure responses to NMDA injected at doses of 4 to 15 nmol, but higher doses (i.e. 20, 25 and 30 nmol) induced fully generalised seizure activity (mean seizure score 4.5 ± 0.5, $n = 4$). The second injection of 3-aminopropylarsonate (10 nmol/0.5 μl significantly reduced ($P < 0.05$) the effect of microinjected NMDA (30 and 40 nmol) to a seizure score of 1.25 ± 0.48 ($n = 4$), and, in addition, it was effective against higher concentrations of NMDA (40 nmol), which are powerfully convulsant.

The same animals were treated with kainate (4 nmol) which was very effective in producing generalised seizure activity at a level of 4.8 ± 0.2 (mean $\pm$ S.E.M.; $n = 5$). The kainate-evoked seizures were not affected by pretreatment with 3-aminopropylarsonate.

4. Discussion

It is very clear from the results that daily focal microinjections into the amygdala of NMDA (4 nmol) led to the development of a fully kindled state after 16.00 ± 1.44 ($n = 6$) days, with a mean seizure score of 4.17 ± 0.40 on the Racine scale. Similar results were reported using lower doses of 2 nmol [13] or by microinjection of higher doses (1.5 μmol) of aspartate or glutamate, or a mixture of both [3,13,26,27].

3-Aminopropylarsonate the arsono analogue of GABA significantly reduced the seizure score of NMDA kindled animals by 78%. The effect of the drug lasted for at least 7 days and was partially reversible after replacing the drug with phosphate buffer microinjection. This relatively long-lasting action of 3-APA on NMDA-induced epilepsy compared to electrically-induced epilepsy is probably due to weaker epileptic-state induced by NMDA.

The effect of 3-aminopropylarsonate was dose dependent since it was gradually reduced following increase in NMDA concentrations, but second injection of the drug reduced the mean seizure score induced by higher doses of NMDA (i.e. 30 to 40 nmol).

The anticonvulsant activity of 3-aminopropylarsonate was selective to NMDA-induced seizures, since it had no effect on kainate-induced seizures even with small doses of the latter (e.g. 4 nmol).

Since NMDA can kindle and induce seizures, and the NMDA receptor antagonists L-AP7, L-AP5 and CPP blocked the chemical and electrical kindling process [3–6,10–13,28,34,35], and NMDA-receptor mediated mechanisms, and probably pre-synaptic mGlu receptors [2], seem to be central in the basic mechanism inducing epilepsy [3–5]. However, 3-aminopropylarsonate is an analogue of GABA and is unlikely to have a direct effect via NMDA receptors.

Thus, it is more likely to be acting by promoting inhibitory GABAergic activity. This may include inhibition of the release of glutamate from glutamatergic neurones. It is well known that inhibitory GABAergic nerve terminals decrease in number at sites of focal epilepsy [3–5,30,31].

The results also show that 3-aminopropylarsonate, the arsono analogue of GABA inhibits the development of electrically induced kindling following an intra-amygdaloid injection of 10 nmol in 0.5 μl phosphate buffer i.e. it inhibits epileptogenesis. It prevented the increase in mean seizure score and augmentation of the afterdischarge duration which normally follows the daily electrical kindling pulse. It also showed anti-convulsant activity in fully kindled animals which was measured as the increase in the afterdischarge threshold (ADT), which is similar to the generalized seizure threshold (GST). The effects on these parameters were reversible, returning to control levels after withdrawal of the drug.

Previous reports have shown that drugs which increase GABA-mediated inhibitory activity, including GABA transaminase (GABA-T) inhibitors, GABA uptake inhibitors and GABA receptor agonists, all delay the rate of development of kindling, and have an anticonvulsant action on the expression of fully kindled seizures [16,18,33,34]. A long-lasting decrease in the inhibitory effect of GABA on hippocampal pyramidal neurones following hippocampal kindling has also been reported [21]. Whether the effect of 3-aminopropylarsonate is due to GABA-mimetic agonist activity, or to enhanced GABA accumulation due to an enzymatic action (e.g., GABA-T or GABA uptake inhibition), is not yet clear and needs further investigation.

However, the phosphono analogue of GABA 3-aminopropylphosphonate is well known as a GABA receptor ligand [32] with partial $GABA_B$ receptor agonist activity [17]. Significantly, the sulfono analogue of GABA (3-aminopropanesulfonic acid) is a potent GABA agonist [14]. Thus, it seems likely that the arsono analogue would have a similar GABA agonist effect. The toxicity of arsono as opposed to arsono-compounds is due to the fact that arsenates are not stable, and enzymes accept arsenate in place of phosphate to incorporate into key compounds such as ATP, causing uncoupling between oxidative metabolism and phosphorylation. Furthermore, there is a

likelihood of producing arsenite which has a high affinity for dithiols such as the dihydrolipoyl groups. This results in inactivation of principal enzymes of oxidative metabolism, such as pyruvate dehydrogenase and oxoglutarate dehydrogenase.

In contrast, the arsono compounds are much more stable than phosphono compounds and they have not yet proved to be good substrates for enzymes that act on the natural amino acids, although they do bind to some of them [20]. The C-P bond clearing enzymes show no or very low activities towards their arsonate analogues [22,23].

In the current experiments the arsono analogue of GABA which was synthesised by Ali and Dixon [1] was investigated. This compound did not appear to be toxic since its anti-epileptogenic and anticonvulsant effects were reversible after 1 week, and the treated animals did not show any visible gross behavioural, physiological or neurological abnormalities 3–6 months after treatment with the drug by multiple intracerebral microinjection. This was judged by their normal patterns of eating, drinking and explorative activities. Though no histological investigations of the amygdala were carried out in this study, previous reports from this laboratory have shown little or no tissue damage after multiple intracerebral injections [10,13]. Further experiments are needed to clarify the mechanism of action of this GABA analogue, but its apparent non-toxicity and high potency qualify it as a potentially clinically useful anticonvulsant.

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