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European Journal of Pharmacology 524 (2005) 53 – 59
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Effect of spermine and N^1-dansyl-spermine on epileptiform activity in mouse cortical slices

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Received 28 June 2005; received in revised form 1 July 2005; accepted 8 September 2005
Available online 25 October 2005

Abstract

N^1-dansyl-spermine is a novel polyamine analogue, which has been demonstrated to have an antagonist action at the stimulatory polyamine site on the *N*-methyl-D-aspartate (NMDA) receptor macrocomplex. Cortical wedges from genetically epilepsy-prone DBA/2 mice demonstrate spontaneous epileptiform activity when perfused with Mg^{2+}-free artificial cerebrospinal fluid (aCSF). This epileptiform activity has been demonstrated to be primarily mediated through the NMDA receptor. N^1-dansyl-spermine reduced the spontaneous epileptiform activity at a high dose (100 μM) but had no effect at a lower dose (50 μM). The polyamine, spermine (300 μM) caused an increase in the rate of the spontaneous epileptiform discharges. This effect of spermine was antagonised by administration of the low dose of N^1-dansyl-spermine (50 μM). This further demonstrates the role of the NMDA receptor in the production of spontaneous epileptiform discharges in the cortical wedge preparation and clearly illustrates both the facilitatory action of spermine and the polyamine antagonist action of N^1-dansyl-spermine at the stimulatory polyamine site on the NMDA receptor.

Keywords: Cortical slice; Epileptiform activity; NMDA receptor; Spermine; N^1-dansyl-spermine; DBA/2 mice

1. Introduction

Epilepsy is a chronic neurological disorder consisting of the unpredictable occurrence of seizures and as yet there is no cure, only a series of symptomatic drug treatments. The drug treatments for epilepsy vary greatly in their mechanisms of action affecting numerous transmitter systems within the brain (for review see Meldrum, 1996). Briefly, the neurotransmitter systems affected in the treatment of epilepsy include gamma-amino-butyric acid (GABA), voltage activated sodium channels, calcium channels and the *N*-methyl-D-aspartate (NMDA) receptor, among others. However, the NMDA receptor and the excitatory amino acid, glutamate, have emerged as one of the primary systems involved in epilepsy.

The NMDA receptor has been widely studied and demonstrated to be involved in many varied cellular mechanisms. It is well established that the NMDA receptor has a role in neuronal degeneration caused by anoxic/ischaemic conditions, seizure mediated brain damage and other neurodegenerative diseases in the brain (Choi, 1985; Rothman and Olney, 1986). The NMDA receptor has also been implicated in the production of seizures in epilepsy. This has been widely illustrated by the production of convulsions in mice by both centrally and peripherally administered NMDA (Czuczwar et al., 1985; Kirby et al., 2004; Moreau et al., 1989; Singh et al., 1990; Turski et al., 1990).

The polyamines, spermine, spermidine and putrescine are a family of di-, tri- and tetra-amines found throughout the body, with high, but locally variable, concentrations in the brain (Al-Deen and Shaw, 1978; Seiler and Schmidt-Glenewinkel, 1975). Previous work has demonstrated a pro-convulsant effect of centrally administered spermine, which is thought to be mediated, at least partially, through the NMDA receptor, with the possible involvement, also, of L-type calcium channels

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doi:10.1016/j.ejphar.2005.09.009

(Anderson et al., 1975; Doyle et al., 2005; Doyle and Shaw, 1996, 1998; Kirby et al., 2004), though other effects such as those on sodium currents (Ran et al., 2003) and kainite receptors (Mott et al., 2003) cannot be ruled out. The polyamines have been widely demonstrated to interact with the NMDA receptor, producing, at physiological concentrations, a potentiation of NMDA activity (Ransom and Stec, 1988; Singh et al., 1990; Sprosen and Woodruff, 1990; Williams et al., 1991). This is thought to be mediated through a stimulatory polyamine site on the extracellular surface of the NMDA receptor macrocomplex. However, high concentrations of polyamines inhibit the activity of the NMDA receptor, demonstrating a dual activity (Sacaan and Johnson, 1990). This led to the theory that there are two extracellular sites on the NMDA receptor macrocomplex, one stimulatory and one inhibitory.

N^1-dansyl-spermine is a novel analogue of the polyamine, spermine, consisting of a polyamine backbone and a single dansyl group at one of the terminal nitrogens. Chao et al. (1997) examined the effects of N^1-dansyl-spermine on cloned NMDA receptors, looking specifically at the inhibitory polyamine site, and found that N^1-dansyl-spermine was a potent blocker of the NMDA receptor by binding to the inhibitory polyamine site (Chao et al., 1997). However, the stimulatory polyamine site is the most relevant physiologically as substantially lower amounts of spermine are required to stimulate the positive site than the negative site. Indeed, it has recently been shown in this laboratory that N^1-dansyl-spermine is a potent polyamine antagonist at the stimulatory polyamine site (Kirby et al., 2004). N^1-dansyl-spermine antagonised both spermine-induced and spermine-enhanced NMDA-induced CNS excitation (Kirby et al., 2004). In addition, N^1-dansyl-spermine has recently been shown to be neuroprotective in the gerbil bilateral carotid artery occlusion model and also in a permanent focal ischaemia model (Kirby and Shaw, 2004; Li et al., 2004).

DBA/2 mice are genetically epilepsy-prone showing susceptibility to audiogenic seizures and as a result have been used in numerous studies examining compounds for potential anti-epileptic activity (Chapman et al., 1984; De Sarro et al., 1999; De Sarro et al., 1988). The cortical wedge preparation from the DBA/2 mouse has been widely used to examine epileptiform activity in vitro. Harrison and Simmonds initially developed the cortical wedge preparation using a brain slice containing cortical efferents (Harrison and Simmonds, 1985). This has since been modified by different groups but is still used to examine the effects of the NMDA receptor on epileptiform activity (Burton et al., 1987; Hu and Davies, 1995, 1997; Naish et al., 2002).

The enhancement of NMDA activity by polyamines has been examined using cortical wedges from a Wistar rat, an experimental set-up similar to that with DBA/2 mice. Robichaud and Boxer demonstrated an enhancement of spontaneous discharge frequency following spermine treatment though higher concentrations inhibited spontaneous discharges (Robichaud and Boxer, 1993). This dual action was similar to that demonstrated by Sacaan and Johnson (1990). Therefore, in this study, it was of interest to examine the effect of N^1-dansyl-spermine on the spontaneous epileptiform discharges in the DBA/2 mouse cortical wedge. Also the effect of N^1-dansyl-spermine was examined on the spermine-induced enhancement of spontaneous epileptiform discharges.

2. Materials and methods

2.1. Subjects

Male and female DBA/2, genetically epilepsy-prone, mice were obtained from Harlan, UK and subsequently bred in the Bioresources unit, Trinity College. Stock mice were housed five to a cage with standard laboratory food and water available ad libitum. The mice were maintained at an ambient temperature of 21 ± 1 °C under a standard 12 h light/dark cycle (light: 7am–7pm). When used the mice were aged between 21 and 42 days.

2.2. Cortical wedge preparation

The method of cortical wedge preparation was similar to that described in Hu and Davies (1995) and is in accordance with the requirements of the current legislation (Cruelty To Animals Act, 1876, European Community Directive, 86/609EC). Briefly, on the day of use the animals were killed by cervical dislocation, decapitated and the brain was quickly removed to ice-cold artificial cerebrospinal fluid (aCSF), which served to remove surplus blood and cooled the tissue. The brain was then transferred to the cutting stage of a McIlwain tissue chopper and secured in place using cyanoacrylate glue (Superglue, Loctite UK). Coronal slices, 500 μm thick, were cut and placed in gassed aCSF at room temperature (20–22 °C). The slices were then separated using fine brushes. From these slices, small wedges of tissue containing cerebral cortex, corpus callosum and striatum were prepared. The wedges were approximately 4 mm in length and 2 mm in diameter at the cortex and reducing to 1 mm at the striatum.

Each cortical wedge was transferred to a two compartment bath, with a silicone grease seal (DC4 electrical compound, Dow Corning) isolating the grey cortical matter from the callosum. The baths were held at a slight angle to allow drainage of the aCSF. The compartment containing the callosal side of the wedge was filled with aCSF and the channel on the cortical side was continuously perfused with gassed aCSF at a flow rate of 2 ml/min. One hour was allowed for the tissue to equilibrate at room temperature. Perfusion of the cortical side of the tissue was then continued with Mg^{2+}-free aCSF (with a corresponding isosmotic increase in Na^+) to facilitate NMDA receptor activation.

2.3. Measurement of electrical activity

The direct current (DC) potential between the two compartments was continuously monitored using silver/silver chloride electrodes. This potential difference was filtered, amplified (Fylde 2601A or ETH-250 from CB Sciences) and recorded on a PowerLab computer system (ADInstruments Ltd).

2.4. Drugs

The following drugs were used: Spermine (Sigma, UK) and N^1-dansyl-spermine (synthesised as previously described (Seiler et al., 1998)). Drugs were only applied to the cortical side of the tissue and were administered dissolved in Mg^{2+}-free aCSF. The normal aCSF contained in millimolar: NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26,

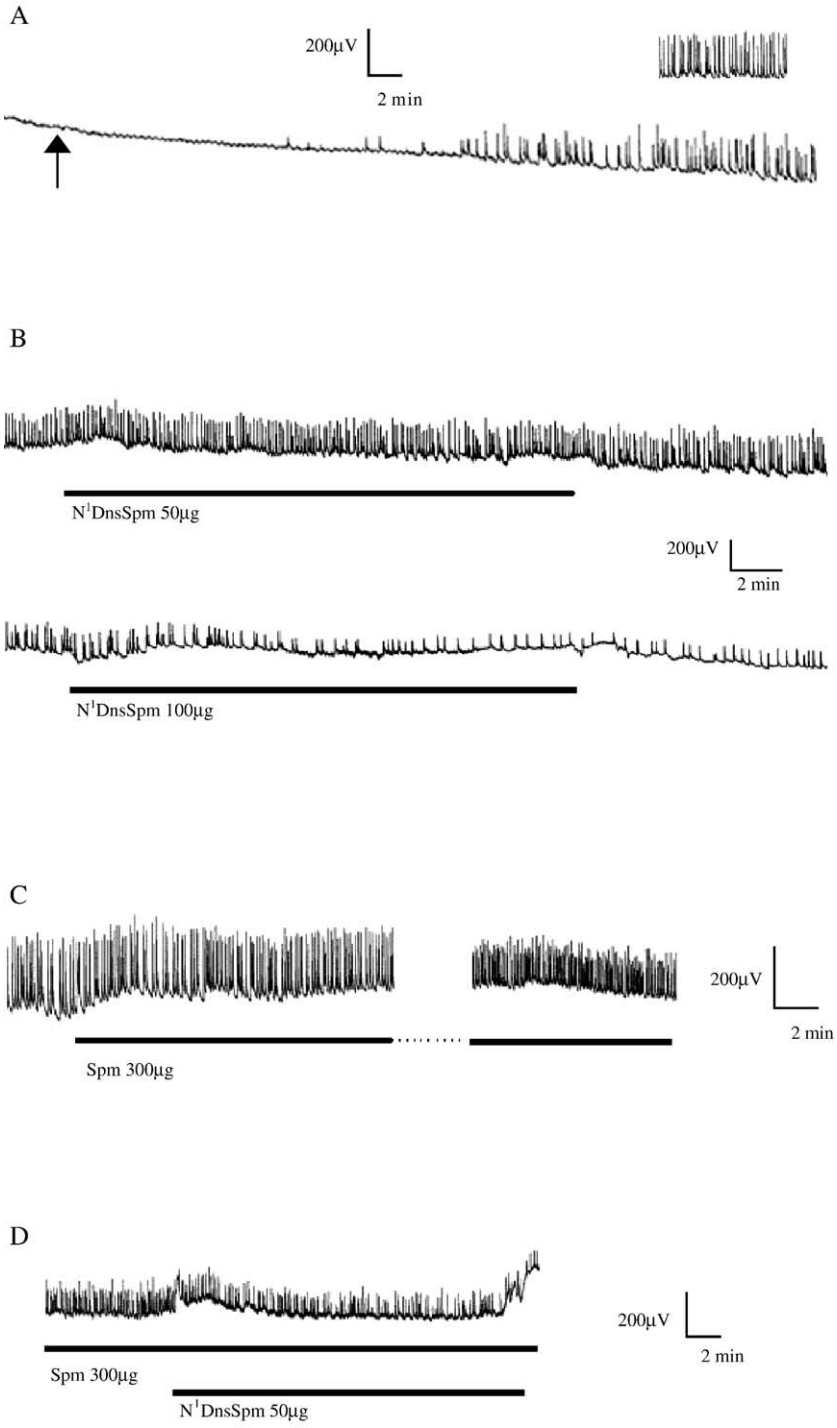

Fig. 1. (A–D) Continuous recordings of spontaneous epileptiform discharges from cortical wedges. (A) Effect of changing perfusion solution from normal aCSF to Mg^{2+}- free aCSF (arrow marks change of perfusion solution). Inset trace shows the effect after 2 h. (B) Effect of two doses of N^1-dansyl-spermine (N^1-DnsSpm)(50 μM—top trace; 100 μM—lower trace) on epileptiform discharges (perfusion period, 20 min, indicated by solid line). (C) Effect of spermine (Spm; 300 μM) on discharges shown immediately after administration and after approximately 90 min. (D) Effect of N^1-dansyl-spermine (50 μM) on spermine enhancement of epileptiform discharges.

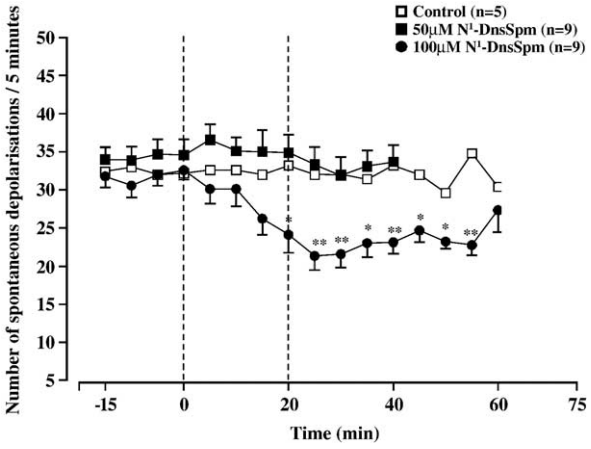

Fig. 2. The effect of treatments of N^1-dansyl-spermine (N^1-DnsSpm) on the number of spontaneous depolarisations per 5 min. The dotted line at time=0 marks the addition of N^1-dansyl-spermine. The second line marks the wash-out with Mg^{2+}-free aCSF. *$P<0.05$, **$P<0.01$, one-way ANOVA vs control. Vertical bars indicate standard error of the means (S.E.M.).

glucose 10 and was maintained at pH 7.4 by gassing with a 95% oxygen/5% carbon dioxide mix. The amount of NaCl was increased in the Mg^{2+}-free aCSF in order to maintain osmolarity.

2.5. Statistical analysis

Magnesium-free aCSF-induced depolarisations became stable after approximately 2 h. Thereafter, the number of depolarisations in the wedge preparation were counted in blocks of 5 min over three periods: 20 min immediately prior to drug perfusion (first period), 20 min drug perfusion (second period) and then 20 or 40 min of drug free perfusion.

Mean values and standard error of the means were calculated and the results presented in graphical form as the number of spontaneous epileptiform discharges per 5 min time block versus time. Differences between groups were assessed using one-way analysis of variance (ANOVA) with repeated measures followed by subsequent analysis of simple main effects. Between group differences were analysed with further ANOVA followed by post hoc Newman–Keuls test. Statistical significance in all tests was accepted at the $P<0.05$ level, with the actual P values shown in the figures.

3. Results

Fig. 1A illustrates a characteristic trace from a cortical wedge and shows the changes that occur when the medium is changed from normal aCSF to Mg^{2+}-free aCSF. After approximately 20 min the first small depolarisations (spike) can be seen and the number increases from there, with time. After 2 h of perfusion with Mg^{2+}-free aCSF, the number of depolarisations becomes stable as can be seen in Fig. 1A inset. Hu and Davies (1997) demonstrated that after 2 h the number of spikes in a 5-min period remained relatively constant (30–40 depolarisations per 5 min). This rate remained stable for up to 6 h (unpublished observations; Hu and Davies, 1997; Srinivasan et al., 1997).

3.1. Effects of N^1-dansyl-spermine on electrical activity

Administration of N^1-dansyl-spermine (50 and 100 μM; $n=9$ in both cases) took place after approximately 2 h, when the rate of discharge had stabilised (Fig. 1B). N^1-dansyl-spermine (50 μM) had no effect on the number of spontaneous epileptiform discharges in each selected 5-min period, when compared to control ($P>0.05$). However, administration of 100 μM N^1-dansyl-spermine had a marked effect on the rate of discharge. The rate was significantly reduced compared to control ($P<0.05$; Fig. 2) and this effect persisted for 35 min following wash-out of the N^1-dansyl-spermine ($P<0.01$ and $P<0.05$; Fig. 2).

3.2. Effect of spermine and N^1-dansyl-spermine

The administration of spermine (300 μM; $n=9$) caused a gradual increase in the rate of discharges, which stabilised after approximately 90 min (Fig. 1C). This is in line with previously published results (Robichaud and Boxer, 1993). The previously ineffective dose of N^1-dansyl-spermine (50 μM) was administered following the protocol mentioned above. At the time of administration of N^1-dansyl-spermine, the rate of discharges had increased by approximately 55–60% over control. This was as a result of continuous administration of spermine. N^1-dansyl-spermine (50 μM; $n=4$–7), perfused in the presence of spermine, caused a significant reduction in the number of spontaneous depolarisations per 5 min when compared to spermine control ($P<0.05$; Fig. 3). This response to N^1-dansyl-spermine was rapid, showing a reduction in the number of discharges, though the difference only reached statistical significance after 20 min. Following wash-out of N^1-dansyl-spermine, the frequency of discharges recovered only

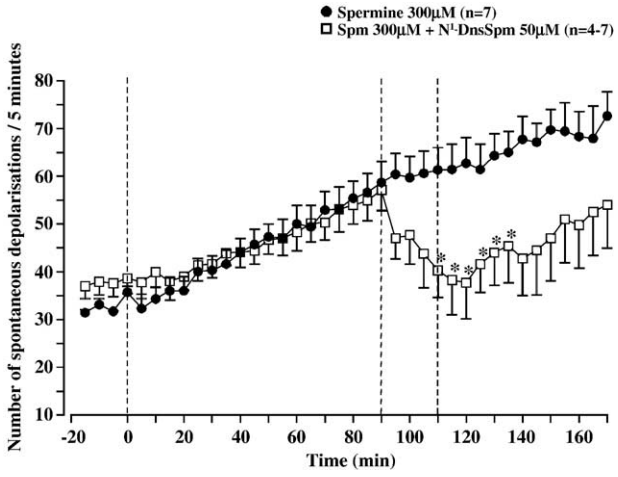

Fig. 3. Effect of 300 μM spermine (Spm) and 50 μM N^1-dansyl-spermine (N^1-DnsSpm) on the number of spontaneous epileptiform depolarisations per 5 min. The dotted line at time=0 represents the initiation of perfusion with Mg^{2+}-free aCSF containing spermine. The lines at 90 and 110 min represent the start and end of drug treatment. *$P<0.05$ one-way ANOVA vs 300 μM Spm alone. Vertical bars indicate S.E.M.

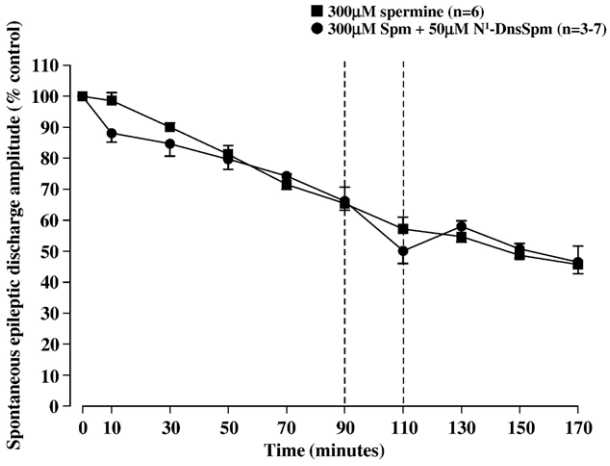

Fig. 4. Effect of spermine (Spm; 300 μM) and N^1-dansyl-spermine (N^1-DnsSpm; 50 μM) on the amplitude of depolarisations. Dotted lines represent addition and wash-out of N^1-dansyl-spermine. Vertical bars indicate S.E.M.

slowly to that of the spermine control, indicating a slow wash-out of N^1-dansyl-spermine from the tissue.

3.3. Effects on amplitude

As is evident from Fig. 1C, the administration of spermine reduced the amplitude of the spontaneous epileptiform depolarisations. As the frequency increased over time so the amplitude decreased (Fig. 4). Indeed, 90 min following administration of spermine the amplitude had decreased to 65% of the initial amplitude. The amplitude continued to decline on administration of N^1-dansyl-spermine but at the same rate and was no different to the control with spermine alone.

4. Discussion

Removal of Mg^{2+} from the perfusion solution caused the production of spontaneous epileptiform discharges in wedges prepared from DBA/2 mice. N^1-dansyl-spermine was demonstrated to have no effect on these discharges at a low dose but to reduce the discharge frequency at a higher concentration. We have also shown that spermine increases the frequency of depolarisations in the cortical wedge and that N^1-dansyl-spermine is effective in reducing the frequency of depolarisations following spermine-induced enhancement. This effect was seen at a dose which had no direct antagonist effect on the NMDA receptor, demonstrating N^1-dansyl-spermine's potential as a polyamine antagonist.

The NMDA receptor is one of the ionotropic glutamate receptors and is blocked in a voltage-dependent manner by magnesium ions. Removal of this magnesium from the perfusion solution allows the NMDA receptor to spontaneously depolarise reaching a steady-state of 30–40 depolarisations per 5-min period, as demonstrated. This, therefore, indicates that NMDA receptor activation is required for discharges to occur. There has however, been some debate over the involvement of other receptor systems (Davies and Shakesby, 1999; Hu and Davies, 1997; Robichaud et al., 1991) but despite the debate, the cortical wedge preparation is used as a model of NMDA receptor-mediated epileptiform activity.

N^1-dansyl-spermine has been demonstrated to be a potent polyamine antagonist at the NMDA receptor, exerting its action through the stimulatory polyamine site (Kirby et al., 2004). The results presented here agree with the previously published data. At a low dose, N^1-dansyl-spermine does not have any effect on the spontaneous epileptiform discharges, suggesting that it does not possess any overt NMDA antagonist activity, as previously shown in an in vivo model in this laboratory (Kirby et al., 2004). However, at the higher concentration there is a reduction in the frequency of discharges. This is most likely due to an agonist action at the inhibitory polyamine site as described by Chao et al. (1997) causing voltage-dependent block of the NMDA receptor. The results demonstrated here compare favourably with the competitive NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl)-propenyl-1-phosphonic acid (CPP) which completely blocked spontaneous depolarisations at 100 μM (Hu and Davies, 1997) and similar findings with dextromethorphan (Naish et al., 2002). The slow recovery of the trace, in the current study, is most likely due to the N^1-dansyl-spermine binding to the cortical wedge and is therefore slowly washed off. This phenomenon has been seen before with other compounds and has been attributed to their lipophilicity (Naish et al., 2002).

As previously mentioned, Robichaud and Boxer (1993) demonstrated, using cortical wedges, that at concentrations up to and including 300 μM spermine a gradual, dose-dependent increase in spontaneous epileptiform discharge frequency was seen. The results here also demonstrate an increase in discharge frequency on perfusion of spermine. A dual action was not seen in this case as the dose used was chosen from the previous study by Robichaud and Boxer (1993), to produce only stimulation.

When the lower dose of N^1-dansyl-spermine was administered to the cortical wedge preparation, following the spermine-increased discharge frequency, there was a rapid reduction in frequency. Since this dose of N^1-dansyl-spermine, on its own, did not have any effect on the discharge frequency, it may be proposed that the effect seen was a polyamine antagonist action of N^1-dansyl-spermine acting through the positive polyamine site on the NMDA receptor macrocomplex. This agrees with previously published data from this laboratory (Kirby et al., 2004) showing a polyamine antagonist action in an in vivo model. As was seen the effect of N^1-dansyl-spermine persists following its removal from the perfusion solution. This persistent action is also described above and can be explained in the same manner.

Another observation that has been shown in a number of previous studies is that of a reduction in amplitude of spontaneous epileptiform discharges in response to treatment with NMDA antagonists and anticonvulsants (Phillips et al., 1997) and also following treatment with spermine or spermidine (Robichaud and Boxer, 1993). The occurrence of a decrease in amplitude coupled with a decrease in frequency (as a result of NMDA antagonist treatment (Phillips et al., 1997)) is most likely as a result of straightforward NMDA receptor blockade. On the other hand, Robichaud and Boxer found a reduction in amplitude coupled with an increase in discharge frequency

(Robichaud and Boxer, 1993). However, an explanation for this phenomenon was not forthcoming. This same reduction in amplitude with increased discharge frequency was found in the present study. One possible explanation for this was put forward by Ong and Kerr (2000). They demonstrated a reduction in amplitude of depolarisations with decreasing concentrations of Ca^{2+}. This suggests the possibility that, in the present study, and that by Robichaud and Boxer, the reduction in amplitude is due to a decrease in Ca^{2+} concentrations within the cortical wedge, possibly as a result of impaired Ca^{2+} homeostasis, though this remains speculative.

Our data provide more evidence to support the use of N^1-dansyl-spermine as a potent polyamine antagonist at the positive polyamine modulatory site on the NMDA receptor macrocomplex. Given this and its neuroprotective properties, N^1-dansyl-spermine is a potentially very useful compound.

Acknowledgements

We would like to thank Ms. Rhona Prendergast for synthesis of N^1-dansyl-spermine, Dr. J. Davies and Dr. W. Marsh for their assistance with the cortical wedge preparation. This work was supported by the Department of Pharmacology, Trinity College Dublin postgraduate award and an Enterprise Ireland award.

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