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Partial adenosine A_1 receptor agonists inhibit sarin-induced epileptiform activity in the hippocampal slice

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Abstract

Organophosphate poisoning can result in seizures and subsequent neuropathology. One possible therapeutic approach would be to employ adenosine A_1 receptor agonists, which have already been shown to have protective effects against organophosphate poisoning. Using an in vitro model of organophosphate-induced seizures, we have investigated the ability of several adenosine A_1 receptor agonists to inhibit epileptiform activity induced by the organophosphate sarin, in the CA1 stratum pyramidale of the guinea pig hippocampal slice. Application of the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) or the partial adenosine A_1 receptor agonists 2-deoxy-N^6-cyclopentyladenosine (2-deoxy-CPA) and 8-butylamino-N^6-cyclopentyladenosine (8-butylamino-CPA) abolished epileptiform activity in a concentration-related manner. The rank order of potency was CPA (IC_{50} 4–5 nM)>2-deoxy-CPA (IC_{50} 113–119 nM) = 8-butylamino-CPA (IC_{50} 90–115 nM). These data suggest that partial adenosine A_1 receptor agonists, which have fewer cardiovascular effects, should be further evaluated in vivo as potential treatments for organophosphate poisoning.

Keywords: Epileptiform; Adenosine; Hippocampal slice; Organophosphate; Sarin; (Guinea pig)

1. Introduction

One consequence of organophosphate poisoning is seizure activity, which is caused by the irreversible inhibition of brain acetylcholinesterase and subsequent cholinergic receptor overstimulation (Shih and McDonough, 1997). As these seizures become resistant to treatment with antimuscarinic drugs alone, therapy usually involves a coadministration of atropine and a benzodiazepine (e.g., diazepam) (Leadbeater et al., 1985; Lallement et al., 1998; Shih and McDonough, 1999; McDonough et al., 2000). An oxime (e.g., pralidoxime; a reactivator of organophosphate-inhibited acetylcholinesterase) is also administered, although it penetrates poorly into the central nervous system (CNS) (see Wolthuis et al., 1994). Although this treatment regimen is generally effective in controlling seizure activity, there is considerable scope for improvement (e.g., diazepam causes respiratory depression and sedation, whilst neuropathy can still occur) (Hayward et al., 1990; Anderson et al., 1997). One possible alternative to diazepam would be an adenosine A_1 receptor agonist (Van Helden and Bueters, 1999).

Adenosine has been shown to have powerful inhibitory actions on neuronal activity and on release of excitatory transmitters (Kirkpatrick and Richardson, 1993; Broad and Fredholm, 1996; Jin and Fredholm, 1997). The selective adenosine A_1 agonist, N^6-cyclopentyladenosine (CPA), has already been shown to protect rats against organophosphate intoxication (Van Helden et al., 1998). Unfortunately, agonists at the adenosine A_1 receptor also have profound effects on heart rate, blood pressure, and body temperature, which have precluded their use as anticonvulsants (for a review of adenosine and seizures, see Dunwiddie, 1999). A possible solution to this prob-

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lem would be the development of adenosine A_1 receptor partial agonists. It has previously been suggested that differences in receptor reserve may allow CNS effects to be maintained (i.e., anticonvulsant activity) without some of the peripheral cardiovascular effects (i.e., reduced heart rate) (Van Helden and Bueters, 1999; Knutsen et al., 1999; Bueters et al., 2003). Two analogues of CPA, 2-deoxy-N^6-cyclopentyladenosine (2-deoxy-CPA) and 8-butylamino-N^6-cyclopentyladenosine (8-butylamino-CPA) have been synthesised (Van der Wenden et al., 1995; Roelen et al., 1996), which are putative A_1 partial agonists and show reduced cardiovascular effects in vivo (Mathot et al., 1995; Van Schaick et al., 1997). In particular, 8-butylamino-CPA has been shown to have a high efficacy in reducing synaptic transmission (an 88% efficacy relative to CPA) compared to its effect on heart rate (a 15% efficacy relative to CPA; Lorenzen et al., 1997).

We have previously described a guinea pig hippocampal slice model in which the irreversible organophosphate anticholinesterase, soman, induces epileptiform activity (Harrison et al., 2000; see also Endres et al., 1989; Apland, 2000). This model may provide the basis for the initial in vitro screening of potential anticonvulsant therapies to replace or supplement those currently used in organophosphate intoxication. The aim of the present study was to evaluate in this slice model the ability of CPA, 2-deoxy-CPA, and 8-butylamino-CPA to terminate bursting induced by the organophosphate anticholinesterase, sarin (this agent was selected in order to facilitate comparisons with parallel work) (Bueters et al., 2002; Bueters et al., 2003).

2. Methods

2.1. Preparation of slices

Male Dunkin–Hartley guinea pigs (250–500 g) were anaesthesised with halothane and killed by decapitation. The brain was removed and transverse hippocampal slices (500 μm thick) were prepared from a block of tissue containing the hippocampus using a vibratome. The slices were stored at room temperature in an artificial cerebral spinal fluid (ACSF) of the following composition (in mM): NaCl 118, KCl 3, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.5, D-glucose 10 (gassed with 95% O_2/5% CO_2). Slices were left to recover for at least 1 h before being transferred to a recording chamber where they were submerged and continuously superfused (10–15 ml/min) with gassed ACSF at 31 °C.

2.2. Extracellular recording

After a further 30 min equilibration, an extracellular recording electrode (filled with 2 M NaCl; 1–5 MΩ resistance) was placed in CA1 stratum pyramidale in order to monitor neuronal activity. Baseline activity was monitored for at least 30 min before the application of 100 nM sarin or soman. Each slice was exposed to the organophosphate for up to 60 min; if epileptiform activity was not present after that time, the slice was classified as unresponsive. Slices unresponsive to sarin or soman had their ability to induce epileptiform activity verified by the addition of 30 μM 4-aminopyridine.

Once epileptiform activity had been established for 15 min, the test compound was added to the reservoir of circulating ACSF. A series of control experiments was also run with 0.03% vol/vol dimethyl sulphoxide (DMSO); this represented the maximum vehicle concentration used.

In a series of separate experiments, immediately following the appearance of sarin-induced bursting, 6.6 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was added to the circulating ACSF, followed 15 min later by 30 nM CPA.

2.3. Data analysis

Data were captured via a CED (Cambridge Electronic Design, UK) 1401 computer interface at 2 kHz using Spike 2 software (Cambridge Electronic Design) throughout the experiment. For the determination of bursts, each recording was individually examined. A burst was defined as a significant deflection from baseline noise (at least twofold over noise), which lasted for at least 100 ms, during which multiple downward deflections were seen. A return to baseline activity for at least 2 s was required before another burst could occur. For the determination of the burst

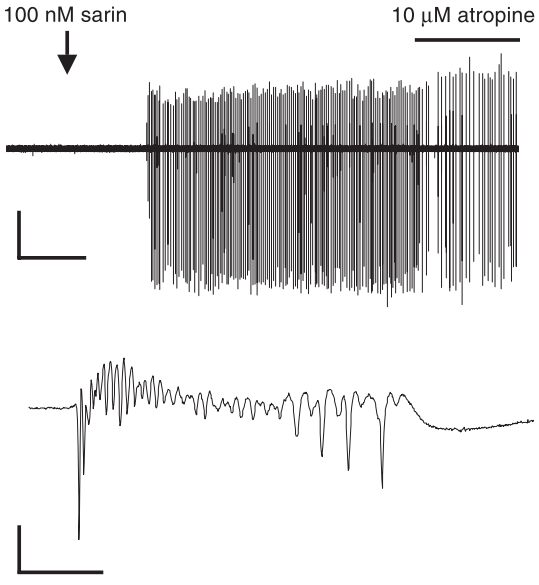

Fig. 1. An example of sarin-induced epileptiform activity is shown on top (horizontal bar: 15 min; vertical bar: 1 mV). An example of a single burst from the upper trace is shown on an expanded time base below (horizontal bar: 100 ms; vertical bar: 1 mV).

amplitude and duration, the burst immediately preceding drug administration (e.g., CPA) for each experiment was analysed using on screen cursors.

Graphs are typically presented using the percentage of pretreatment burst rate (i.e., the average number of bursts recorded per minute during the 10–20 or 30–40 min epoch as a percentage of the rate for the 10-min period before drug application). These two time points post application were chosen in order to potentially highlight any differences in the diffusion/equilibration of compounds.

In order to facilitate comparisons (and allow calculation of the IC_{50} and Hill slope), data were fitted to a sigmoid dose–response curve (variable slope, with the bottom of the curve constrained to zero and the top constrained to the value obtained from controls) and the lines of best fit plotted (Graphprism, v. 3.0; GraphPad Software, San Diego, CA, USA).

2.4. Drugs

All chemicals were obtained from Sigma-Aldrich (Poole, UK) with the following exceptions: sarin (isopropyl methylphosphonofluoridate) and soman (pinacolyl methylphosphonofluoridate) (purity >95%) were synthesised on site by the Chemistry Department of Dstl (Porton Down, UK); 2-deoxy-CPA and 8-butylamino-CPA were synthesised at the Leiden University (The Netherlands).

Sarin and soman were diluted daily from a ≈ 5 mg/ml stock (isopropyl alcohol) using ACSF to produce a final concentration of 100 nM. Test compounds were made up into a stock solution and frozen into aliquots. A fresh aliquot was diluted as necessary and used for each experiment. Tested compounds were dissolved as follows: CPA, 2-deoxy-CPA, and 8-butylamino-CPA in DMSO; atropine sulphate in water; DPCPX in ethanol; and 4-aminopyridine in water.

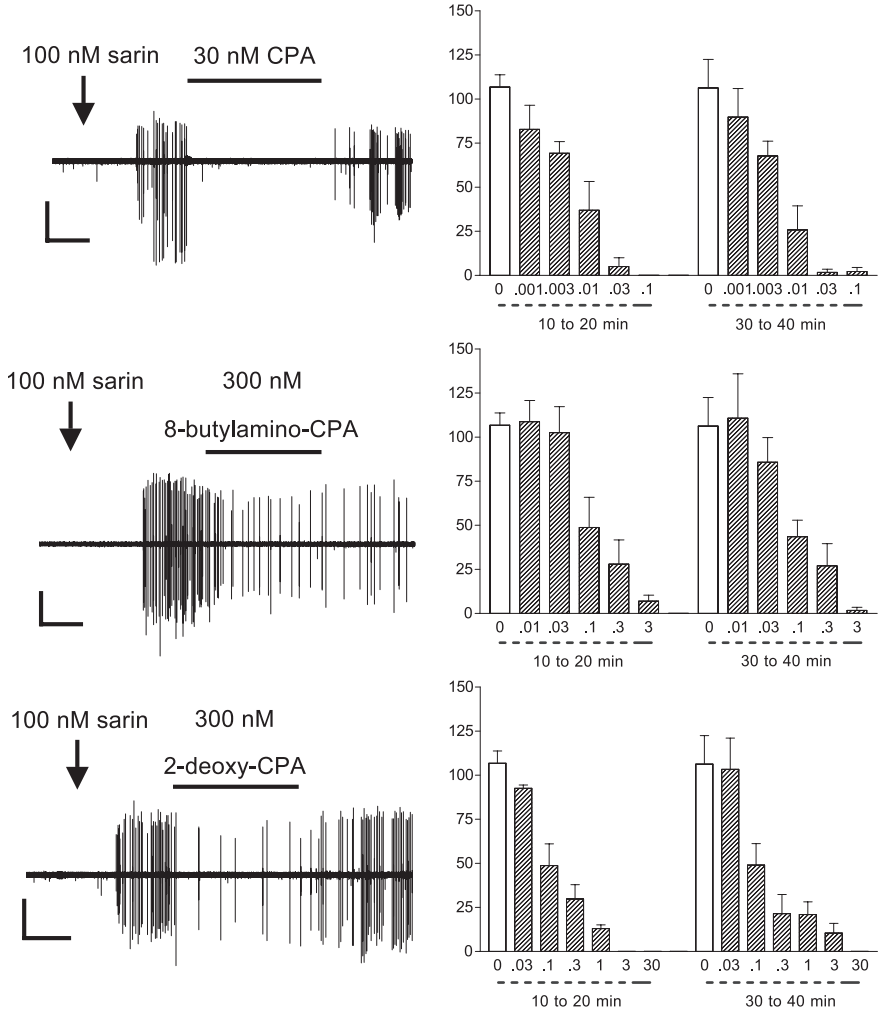

Fig. 2. Examples of the effect of adenosine A_1 receptor agonists on sarin-induced bursting are shown on the left (horizontal bar: 15 min; vertical bar: 1 mV). The concentration-related effect upon bursting 10–20 and 30–40 min after the application of agonists is shown right (abscissa: concentration in micromolar; ordinate: burst rate as a percentage of pretreatment). Each bar represents the mean response (± S.E.M.) from four slices. A significant reduction in burst rate ($P \leq 0.05$) was seen for CPA at ≥ 10 nM, for 8-butylamino-CPA at ≥ 100 nM, and for 2-deoxy-CPA at ≥ 100 nM (one-way ANOVA with Dunnett's post-hoc test) compared to control values.

3. Results

3.1. Application of organophosphates

Application of 100 nM sarin to slices induced epileptiform activity within 60 min in 89% of preparations. In the 69 slices studied, the average latency to onset of bursting was 20.3 ± 1.4 min (mean ± S.E.M.), with an initial bursting frequency of 1.9 ± 0.1 bursts/min, an amplitude of 2.95 ± 0.2 mV, and a duration of 826 ± 85 ms (bursts within a single recording were fairly consistent). Discharges consisted of trains of low-amplitude spikes superimposed on a positive-going field potential (Fig. 1), similar to those seen previously with soman. In concordance with previous work (Harrison et al., 2000), sarin-induced bursting could be partially inhibited by the muscarinic antagonist, atropine (10 μM; Fig. 1). This bursting could also be abolished by the AMPA/kainate antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (5 μM; data not shown).

In order to allow a better comparison with our previous work, a brief series of experiments using the organophosphate, soman (100 nM), was carried out. In the 19 slices studied, the average latency to onset of soman-induced bursting was 20.1 ± 2.8 min (mean ± S.E.M.), with an initial bursting frequency of 2.0 ± 0.2 bursts/min, an amplitude of 1.7 ± 0.2 mV, and a duration of 604 ± 39 ms.

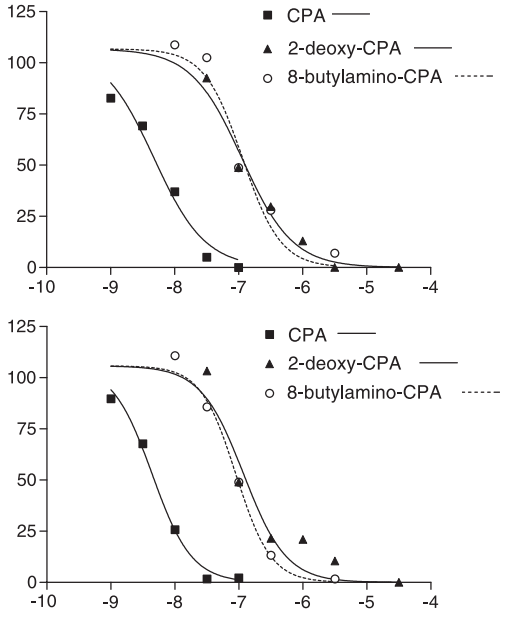

Fig. 3. A comparison of the effects of CPA, 2-deoxy-CPA, and 8-butylamino-CPA on sarin-induced bursting. The effect of each compound 10–20 min postapplication is shown on top whilst the effect 30–40 min postapplication is shown in the bottom (abscissa: concentration log *M*; ordinate: burst rate as a percentage of pretreatment). The lines represent curves of best fit (fitted to a sigmoid dose–response curve, variable slope with bottom constrained to zero and the top constrained to the vehicle control value) whilst symbols represent the mean data points ($n = 4$ in each case). Error bars were omitted in order to improve clarity.

Table 1
The derived IC_{50} and hill slope values for CPA, 2-deoxy-CPA, and 8-butylamino-CPA for antagonism of sarin-induced bursting

Compound	10–20 min post treatment		30–40 min post treatment	
	IC_{50} (nM)	Hill slope	IC_{50} (nM)	Hill slope
CPA	4.8	– 1.09	4.4	– 1.40
2-Deoxy-CPA	113	– 1.10	119	– 1.30
8-Butylamino-CPA	115	– 1.46	90	– 1.58

The table summarises the effect of each adenosine A_1 receptor ligand on sarin-induced bursting. In order to obtain the results above, each data set was fitted to a sigmoidal dose–response curve (variable slope, with the bottom constrained to zero and the top constrained to the vehicle control value), shown in Fig. 3, and the data extracted through Graphprism.

There was no significant difference in the latency and burst rate induced by the two organophosphates ($P \geq 0.05$, unpaired *t* test), although a significant difference was detected between the amplitude ($P \leq 0.001$, unpaired *t* test with Welch's correction for unequal variance) and the duration ($P \leq 0.05$, unpaired *t* test with Welch's correction).

3.2. Effect of CPA, 2-deoxy-CPA, and 8-butylamino-CPA on organophosphate-induced bursting

The ability of each adenosine A_1 receptor agonist to inhibit sarin-induced epileptiform activity was investigated. All compounds tested were found to inhibit bursting in a concentration-related manner (an example of each and the effect of varying concentrations are shown in Fig. 2). In order to facilitate comparisons, fitted sigmoid dose–response curves are shown in Fig. 3 (see also Table 1). Overall, the experiments showed little difference between the effect of the adenosine agonist 10–20 min after application compared to 30–40 min after application, with compounds tending to have a slightly greater effect at the later time point.

Significant reductions in the burst rate [$P \leq 0.05$, one-way analysis of variance (ANOVA) with Dunnet's post-hoc test] were seen with CPA at concentrations ≥ 10 nM and with 2-deoxy-CPA and 8-butylamino-CPA at concentrations ≥ 100 nM, with the derived IC_{50} values suggesting an overall rank order potency of CPA>2-deoxy-CPA = 8-butylamino-CPA.

Additionally, it was noticed that although the effects of CPA and 2-deoxy-CPA could be removed by washing, bursting was only poorly re established following the removal of 8-butylamino-CPA.

The application of CPA inhibited soman-induced bursting in a concentration-related manner (Fig. 4), with a significant reduction seen at concentrations of ≥ 10 nM ($P \leq 0.001$, one-way ANOVA with Dunnet's post-hoc test). A comparison of the effects of 10 nM CPA on sarin- and soman-induced bursting showed little difference. The burst rate 10–20 min after 10 nM CPA application was 37.0 ± 16.3% of pretreatment in sarin-induced slices and

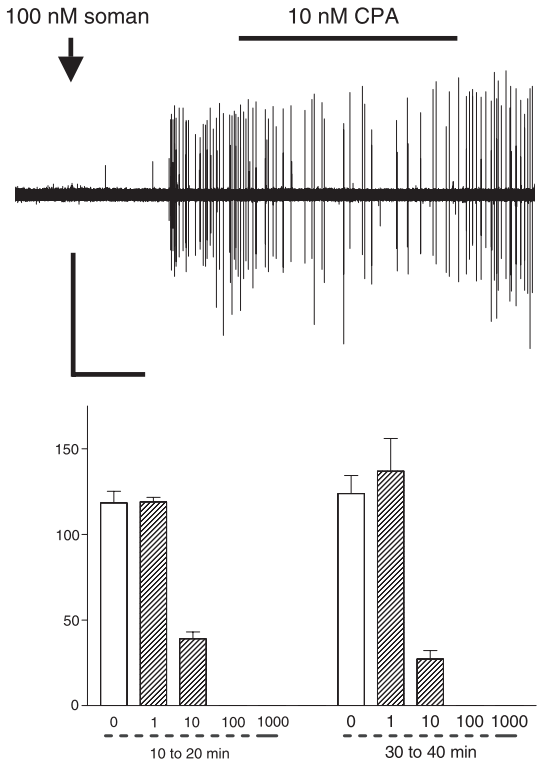

Fig. 4. The effect of selective adenosine A_1 receptor agonist, CPA, on soman-induced bursting. An example of the effect of CPA is shown on top (horizontal bar: 15 min; vertical bar: 1 mV). The concentration-related effect upon bursting 10–20 and 30–40 min after the application of agonists is shown at the bottom (abscissa: concentration in nanomolar; ordinate: burst rate as a percentage of pretreatment). Each bar represents the mean response (± S.E.M.) from three to four slices. A significant reduction in burst rate compared to control was seen at concentrations ≥10 nM ($P \leq 0.001$, one-way ANOVA with Dunnett's post-hoc test).

39.0 ± 4.1% in soman-induced slices. The burst rate 30–40 min after 10 nM CPA application was 25.8 ± 13.7% of pretreatment in sarin-induced slices and 27.3 ± 4.9% in soman-induced slices.

3.3. The effect of DPCPX

To determine whether the effects of CPA and the two CPA analogues were being mediated by adenosine A_1 receptors, we hoped to reverse the inhibition with the selective adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine. However, the application of adenosine A_1 receptor antagonists, including DPCPX, has been shown to induce epileptiform activity in guinea pig hippocampal slices (Alzheimer et al., 1989). In an attempt to avoid this problem, we used DPCPX at a concentration of 6.6 nM (added immediately upon the onset of bursting); this concentration is below that which induces epileptiform activity and corresponds to twice the pA_2 value in the guinea pig hippocampal slice (Alzheimer et al., 1991). Thus, 30 nM CPA in the presence of 6.6 nM DPCPX should produce the same inhibition as 10 nM CPA.

In this separate series of experiments (a total of six slices), the sarin-induced bursting had a latency of 16.9 ± 2.4 min to onset, a burst rate of 3.2 ± 0.3 bursts/min, an amplitude of 2.2 ± 0.3 mV, and a duration of 562 ± 27 ms (mean ± S.E.M.). These data were significantly different from the other sarin-treated slices, in terms of both the burst rate ($P \leq 0.001$, unpaired t test) and duration ($P \leq 0.001$, unpaired t test with Welch's correction) but not latency ($P \geq 0.05$, unpaired t test) or amplitude ($P \geq 0.05$, unpaired t test with Welch's correction).

The burst rates observed 10–20 min after application were 16.0 ± 6.0%, 37.0 ± 16.3%, and 5.0 ± 5.0% of pretreatment levels (for 30 nM CPA + DPCPX, 10 nM CPA, and 30 nM CPA, respectively).

The burst rates observed 30–40 min after application were 43.6 ± 12.2%, 25.8 ± 13.7%, and 1.8 ± 1.8% of pretreatment levels (for 30 nM CPA + DPCPX, 10 nM CPA, and 30 nM CPA, respectively).

4. Discussion

Organophosphate poisoning by an accidental exposure to pesticides, or by a deliberate attack using nerve agents is a serious problem. The current treatment regime, whilst generally effective, has room for improvement. One possible solution to this problem is the development of partial adenosine A_1 receptor agonists. Because tissues have different receptor reserves, a compound with a lower efficacy might exert a full effect in one tissue but a seemingly insignificant effect in another. Furthermore, because receptors can interact with a number of different effectors (G_i/G_o, etc.), the drug–receptor complex might preferentially affect one transducing system (Kenakin, 1995). Thus, it might be possible for an adenosine agonist to maintain anticonvulsant efficacy but lack the undesirable cardiovascular effects (Lorenzen et al., 1997).

Application of the organophosphate anticholinesterase, sarin, induced epileptiform activity in 89% of guinea pig hippocampal slices. The principal stimulus for epileptiform activity is likely to be the excitatory effect of acetylcholine on hippocampal neurones, primarily the inhibition of certain K^+ channels (Bernado and Prince, 1982; Cole and Nicholl, 1984; Muller and Misgeld, 1986). However, why 11% of slices failed to respond is uncertain (epileptiform activity was seen with a subsequent application of 4-aminopyridine). It has previously been reported that the organophosphate, paraoxon, induces epileptiform activity in ≈ 70% of slices (Endres et al., 1989), whilst injection of the irreversible acetylcholinesterase inhibitor, VX, into the amygdala induced convulsions in 67% of animals (McDonough et al., 1987) and injection of carbachol into piriform cortex induced clonic seizures in 62% of animals (Piredda and Gale, 1985). It thus seems likely that in some circumstances, cholinergic stimulants are 'weaker' drivers of seizure activity than

other agents. Moreover, as cholinergic innervation varies across the hippocampus (Lewis et al., 1967; Kasa, 1986) as does the propensity for epileptiform activity (Bragdon et al., 1986), it is tempting to speculate that a combination of the above factors may explain why only a proportion of slices responds to sarin.

As expected, the sarin-induced activity was sensitive to atropine and was almost identical in rate and latency to that produced by soman (e.g., burst rate 1.9 ± 0.1 and 2.0 ± 0.2 bursts/min; latency to onset 20.3 ± 1.4 and 20.1 ± 2.8 min, for sarin and soman, respectively). These values also correlate well with previous work (Harrison et al., 2001). Thus, the epileptiform activity seen here is almost certainly a result of cholinergic receptor overstimulation (via accumulation of acetylcholine due to the irreversible inhibition of acetylcholinesterase) and not through some alternative mechanism of sarin or soman (Bakry et al., 1988; Lau et al., 1988; Rocha et al., 1998; Niijima et al., 1999). This is also supported by the finding that 10 nM CPA produces the same inhibition independent of whether bursting is induced by sarin or soman. However, it was noted that the soman- and sarin-induced bursting was significantly different in terms of the amplitude and duration of bursts. This difference was unexpected and requires further investigation, although it supports the idea that organophosphates might be exerting additional effects in the slice (Santos et al., 2003).

It has previously been shown that treatment of rats with the selective adenosine A_1 receptor agonist, CPA, can protect against organophosphate intoxication (Van Helden et al., 1998). Here, CPA was able to abolish the organophosphate (sarin and soman)-induced epileptiform activity. Furthermore, the IC_{50} of <5 nM corresponds well with the previously reported IC_{50} of >3 nM for abolishing the population spike in the guinea pig hippocampal slice (Alzheimer et al., 1991). The ability to inhibit sarin-induced bursting was also shared by the proposed partial adenosine A_1 receptor agonists 2-deoxy-CPA and 8-butylamino-CPA, with IC_{50} values of $\approx 113-119$ and $\approx 90-115$ nM, respectively. These values and their rank order of potency are similar to other reports (Lorenzen et al., 1997; Bueters et al., 2003), although discrepancies may be explained by the different species used (rat vs. guinea pig) and by the paradigm used (inhibition of field potentials vs. epileptiform activity). Interestingly, the work by Lorenzen et al. using a rat hippocampal slice model reported IC_{50} values of 12.9 and 2770 nM for CPA and 8-butylamino-CPA, respectively, for the suppression of synaptic transmission in the hippocampus (a relative difference in potency of ≈ 200 fold, compared to ≈ 20 fold seen here). Moreover, this effect was easily reversed following washing.

We found that interpretation of the 8-butylamino-CPA data was complicated by the suspicion of an additional unknown effect, a suspicion also reported by Bueters et al., 2003. For instance, the effects of 8-butylamino-CPA were only poorly washed off (see Fig. 3). Such an additional effect would also explain the discrepancy in the effects of 8-butylamino-CPA: generally, increasing the alkyl chain length (i.e., 8-methyl-CPA → 8-ethyl-CPA, etc.) causes a reduction in efficacy of inhibition of hippocampal synaptic transmission (Lorenzen et al., 1997), but they found that 8-butylamino-CPA was an exception, producing a maximal effect not significantly different from the full agonist CPA.

An obvious means to demonstrate that the observed effects are through adenosine A_1 receptor activation alone would be through addition of a selective antagonist (e.g., 8-cyclopentyl-1,3-dipropylxanthine). Unfortunately, the application of adenosine A_1 receptor antagonists can itself induce epileptiform activity in rat and guinea pig hippocampal slices (Ault and Wang, 1986; Alzheimer et al., 1989; but see Chesi and Stone, 1997), presumably due to the loss of endogenous adenosine inhibition (Dunwiddie and Diao, 1994). Even employing concentrations of DPCPX below that required to induce epileptiform activity (i.e., less than 30 nM) caused a significant change in the sarin-induced bursting. Consequently, such an approach is not feasible, as the epileptiform activity is no longer a sarin-induced phenomenon.

Overall, therefore, the organophosphate, sarin, induces epileptiform activity in the guinea pig hippocampal slice almost identically to that with soman. This activity can be abolished by the adenosine A_1 receptor agonists CPA, 2-deoxy-CPA and 8-butylamino-CPA. Thus, both 2-deoxy-CPA and 8-butylamino-CPA, which act as partial agonists in cardiovascular models (compared to CPA), act as full agonists in this central preparation. Interestingly, the results with 8-butylamino-CPA suggest that similar compounds may show a preference for suppressing epileptiform activity over synaptic transmission. This opens up the possibility that A_1 receptor agonists may be able to have anticonvulsive activity without suppressing normal neurotransmission (and so less side effects). These data support the idea that novel partial agonists, which have fewer cardiovascular effects, may be efficacious in terminating seizure activity and are a promising avenue of approach for new treatments in organophosphate poisoning. Further experiments need to be performed in order to determine whether adenosine A_1 receptor partial agonists show the same antiseizure potential in vivo as they do in vitro.

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