THE ANTICONVULSANT ACTIONS OF NOVEL 'BROAD-SPECTRUM' Ca^{2+} CHANNEL BLOCKERS AND LOW AFFINITY, UNCOMPETITIVE NMDA RECEPTOR ANTAGONISTS

by

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ABSTRACT

Epilepsy is a prominent neurological disorder. Presently available anticonvulsant drugs however fail to alleviate seizures in approximately 25% of individuals, and are often accompanied by serious side effects. More efficacious and less toxic agents are required. In this study, the effects of a range of structurally dissimilar σ site ligands were examined against evoked and spontaneous epileptiform activity induced in rat hippocampal slices by perfusion with Mg^{2+}-free medium. Extracellular recordings were made in the CA1 hippocampal region of epileptiform activity evoked by stimulation of the Schaffer collateral (SC) pathway, and of spontaneous epileptiform activity originating from the CA3 hippocampal region. Evoked and spontaneous epileptiform activity was inhibited by all compounds tested with the rank order (IC_{50} values against evoked epileptiform activity in μM): dextrorphan (2) > ifenprodil (6) > dextromethorphan (10) > 1,3-di(2-tolyl)guanidine (15) > loperamide (28) > carbetapentane (38) > caramiphen (46) > opipramol (52). Ifenprodil, loperamide, caramiphen and dextrorphan were also examined for their effects on the input/output (I/O) functions along the SC pathway and on the paired pulse facilitation (PPF) ratio. An effect was observed only in the presence of caramiphen, which showed a decrease in the synaptic transmission I/O function and reduced markedly the PPF ratio. The (micromolar) concentrations required for the anticonvulsant activity of the σ ligands tested suggests that their anticonvulsant actions are not mediated by high affinity (nanomolar) binding to σ binding sites, but rather to blockade of high voltage activated Ca^{2+} channels and/or NMDA receptors, actions which occur at micromolar concentrations.

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INTRODUCTION

Epilepsy

Epilepsy currently stands as the most prevalent neurological disorder, afflicting almost two percent of the population. Additionally, approximately one in 20 people will experience an epileptic type seizure at some point in their lifetime (Jefferys, 1990; McNamara, 1992; Perucca, 1993; McNamara, 1994). Although a number of pharmacological therapies have been developed for recurrent seizures, about 25% of patients still remain refractory to these current medications (Porter and Rogawski, 1992; Wilder, 1995). Many of the currently available drugs can also induce an array of serious side effects, such as encephalopathy and systemic lupus erythematosus (phenytoin), blood dyscrasias (carbamazepine) and liver damage (sodium valproate). More efficacious and less toxic anticonvulsant compounds are thus required.

Over the past few decades, our knowledge of the cellular and molecular mechanisms underlying epileptic seizures has increased. It is now apparent that normal brain function depends upon a delicate balance between excitatory and inhibitory inputs and that any disruption in this balance which acts to increase excitation or decrease inhibition can result in seizure activity (McNamara, 1992; Porter and Rogawski, 1992; Dichter, 1994). A seizure is characterized by a large depolarization of a population of neurons, accompanied by synchronous high frequency firing, resulting in abnormal brain function (Prince and Connors, 1986; Taylor, 1988; McNamara, 1992). This seizure activity arises as a function of processes occurring at the level of individual cells, compounded by the synchronous activity of the neurons as a population.

At the cellular level, abnormal activity of either excitatory or inhibitory mechanisms can give rise to seizures. Contributing most significantly to the excitatory component are the excitatory amino acid receptors, which consist of the *N*-methyl-D-aspartate (NMDA), the non-NMDA (*i.e.* (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate) and the metabotropic glutamate receptor (mGluR) subtypes. Of primary importance is the NMDA receptor-channel complex, which has been shown to play a major role in the initiation and propagation of seizure activity, with both competitive and noncompetitive NMDA receptor antagonists demonstrating anticonvulsant activity in numerous animal epilepsy models and human clinical trials (Dingledine *et al.*, 1990; Chapman, 1991; Rogawski, 1992; see below). More recently, non-NMDA receptors, which were thought only to mediate 'fast' excitatory neurotransmission during normal physiological functioning, have also been found to participate in seizure activity (Dichter, 1994; Meldrum, 1994). Competitive antagonists for the AMPA receptor such as 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) possess anticonvulsant activity in various animal models. However, as these receptors are extensively involved in 'fast' synaptic transmission under non-pathological conditions, the clinical therapeutic benefit of AMPA receptor antagonists remains to be seen (Meldrum, 1994; Muir and Lees, 1995). Finally, the most recent findings also suggest a contribution of mGluRs to seizure activity (*e.g.* McDonald *et al.*, 1993; Thomsen *et al.*, 1994).

Given that activation of postsynaptic glutamate receptors is central to the development and propagation of seizure activity, it therefore follows that mechanisms which act upon the release and subsequent re-uptake of glutamate could also participate in epileptogenesis. Glutamate is removed from the synaptic cleft by a Na^+-dependent, high-affinity glutamate

uptake carrier, located both on neurons and glial cells. It is thought that glutamate's action is terminated only once it has been removed from the synaptic cleft; therefore any malfunction in this uptake mechanism could possibly result in increased excitation (Greenamyre and Porter, 1994). However, there exists a lack of published evidence concerning a link (if any) between the glutamate uptake carrier and seizure activity, and its role in seizure disorders, if any, remains to be seen.

Excessive glutamate release, under the influence of presynaptic voltage-sensitive calcium (Ca^{2+}) channels (VSCCs), has also been suggested to contribute to epileptic phenomena (Meldrum, 1994). The most recent evidence suggests that central nervous system (CNS) glutamate release is mediated by a combination of N-, P- and Q-type VSCCs (Wheeler *et al.*, 1994a & 1994b; Miljanich and Ramachandran, 1995). As the localization and pharmacological characterization of these VSCCs continues to undergo investigation, it is difficult to assess whether they might contribute to excess glutamate release during seizure activity and thereby present a viable target for pharmacological intervention. However, the therapeutic potential of presynaptic VSCC blockers certainly looks promising as evidenced by studies which have demonstrated the anticonvulsant activity of relatively specific blockers of P- and Q-type Ca^{2+} channels (*e.g.* Robichaud *et al.*, 1994). In addition, the anticonvulsant actions of adenosine (Ault and Wang, 1986) appear likely to reflect its ability to block presynaptic (particularly N-type) VSCCs (Scholz and Miller, 1991; Mogul *et al.*, 1993; Yawo and Chuhma, 1993) and, in this way, decrease glutamate release (*e.g.* Prince and Stevens, 1992; Wu and Saggau, 1994a). Indeed, it has been suggested that adenosine may act as the body's endogenous antiepileptic agent (Dragunow, 1991).

As well as being involved in neurotransmitter release, VSCCs are also found postsynaptically and can contribute to excitation of the postsynaptic cell. Postsynaptic VSCCs include primarily the dihydropyridine (DHP) sensitive L-type channel and the low voltage activated T-type channel, although N- and P-type channels have also been identified postsynaptically (Gohil *et al.*, 1994; Kimura *et al.*, 1995). Upon postsynaptic depolarization, these channels contribute to the Ca^{2+} bursting characteristic of epileptiform activity (Heinemann and Hamon, 1986; Prince and Connors, 1986). Evidence for their involvement in seizure discharge arises from investigations using both animal and human tissue which have found that compounds acting to inhibit Ca^{2+} flux through these Ca^{2+} channels demonstrate anticonvulsant activity (DeSarro *et al.*, 1991; Mutani *et al.*, 1991). Furthermore, many of the anticonvulsant drugs currently available such as phenytoin, ethosuximide and the barbiturates include Ca^{2+} channel blocking activity as part of their spectrum of actions (Rogawski and Porter, 1990; see below). In addition to their Ca^{2+} channel blocking actions, certain clinically effective anticonvulsant drugs also possess Na^{+} channel blocking properties, suggesting a role for voltage-dependent Na^{+} channels in convulsant activity. Examples of these drugs include phenytoin, carbamazepine and valproate, all currently used antiepileptic compounds (Wilder, 1995).

Although excitatory ionic conductances appear to play a central role in the initiation and propagation of seizure discharges, under normal conditions inhibitory synaptic transmission acts to counterbalance the excitatory influences and a reduction in inhibitory drive *per se* can also lead to the development of seizure discharges. Indeed, numerous animal models of epilepsy have been developed simply by blocking inhibitory conductances, demonstrating the essential role synaptic inhibition normally plays in suppressing seizure

activity (Schwartzkroin, 1986; Engel, 1989). The principal inhibitory neurotransmitter of the mammalian CNS is γ-aminobutyric acid (GABA), which acts at both $GABA_A$ and $GABA_B$ receptors. At $GABA_A$ receptors, GABA opens chloride (Cl^-) channels, resulting in a postsynaptic hyperpolarization. Activation of pre- and postsynaptic $GABA_B$ receptors results in a K^+ channel mediated hyperpolarization, which presynaptically acts to inhibit subsequent GABA release (*e.g.* Gähwiler and Brown, 1985). The first antiepileptic drugs to be developed, such as the barbiturates and later the benzodiazepines, targeted the GABA system, acting to increase the Cl^- conductance through the $GABA_A$ receptor-operated channel (Dichter, 1989; Löscher, 1993). More recently, other approaches have been taken to enhance $GABA_A$-mediated inhibition. For example, vigabatrin produces a prolonged elevation of GABA levels in brain by inhibiting GABA aminotransferase (the only significant pathway for degradation of brain GABA) and is an effective anticonvulsant agent (see Upton, 1994).

Although ionic conductances, acting at the cellular level, obviously participate in the genesis and propagation of epileptic phenomena, a seizure discharge itself reflects the synchronous activity of a population of neurons, *i.e.* it is a network phenomenon. Many studies have examined possible mechanisms whereby entire populations of neurons can be recruited into synchronous bursting activity. Findings from these investigations indicate that non-synaptic, as well as synaptic, interactions can participate in the synchronization of neuronal activity (see Dudek *et al.*, 1986). Examples of non-synaptic mechanisms which play a role in synchronizing neuronal populations include: ephaptic effects, electrotonic coupling through gap junctions and changes in the extracellular concentrations of potassium (K^+) and Ca^{2+} ions (Yaari and Jensen, 1988; Jefferys, 1990).

Ephaptic (or electric field) effects occur when electrical currents generated by active neurons excite neighbouring neurons. Ephaptic interactions are especially prominent in structures whose neurons are tightly packed, with little extracellular space, characteristics of the densely laminated hippocampus, where ephaptic effects have been observed both *in vitro* and *in vivo* (Prince, 1988).

Electrotonic coupling involves the transfer of current through specific channels in gap junctions which connect the cytoplasm of adjacent cells. Gap junctions have been identified in the hippocampus both electrophysiologically (*e.g.* MacVicar and Dudek, 1982) and by the injection of the fluorescent dye Lucifer yellow (*e.g.* Church and Baimbridge, 1991; Perez-Velaquez *et al.*, 1994). Recent data have suggested the involvement of gap junctions in a number of *in vitro* seizure models (*e.g.* Church and Baimbridge, 1991; Perez-Velazquez *et al.*, 1994), but there exists little experimental evidence either supporting or refuting a role for gap junctions in epileptogenesis *in vivo*. Also, previous electrophysiological estimates of the resistance of the connections and computer simulations reflecting these estimates have suggested that the role of electrotonic interactions via gap junctions in synchronizing large populations of neurons may be fairly minimal (Jefferys, 1990).

Seizure activity has been shown to be accompanied by both an increase in extracellular K^+ and a decrease in extracellular Ca^{2+} concentrations (Heinemann and Louvel, 1983; Dichter and Ayala, 1987; Prince, 1988). These changes in extracellular ion levels result in a depolarizing effect, serving to bring the neurons closer to threshold and enhancing the synchronization of neurons within a population. These ionic alterations certainly seem to contribute significantly to epileptogenesis as convulsant activity can be induced simply by bathing neural tissue in media containing either high K^+ concentrations (*e.g.* Rutecki *et al.*,

1985; Traynelis and Dingledine, 1988), low Ca^{2+} concentrations (*e.g.* Haas and Jefferys, 1984) or a combination of high $[K^+]$ and low $[Ca^{2+}]$, forming the basis of the commonly used high $[K^+]$ and/or low $[Ca^{2+}]$ models of epilepsy (see below).

In summary, epilepsy is a complex phenomenon involving various synaptic and non-synaptic components as briefly outlined above. Many of these mechanisms present potential targets for therapeutic pharmacological intervention. In the present work, I have focused on the possibility that compounds with inhibitory actions at both the NMDA receptor-channel complex and at VSCCs, specifically high voltage-activated (HVA) types, may possess novel anticonvulsant activities. The characteristics of the NMDA receptor-channel complex and HVA Ca^{2+} channels will therefore be described in further detail.

The NMDA receptor-channel complex

The NMDA receptor is one of three subtypes of ionotropic excitatory amino acid receptors which exist in the mammalian CNS. It is named after its preferred pharmacological agonist, *N*-methyl-D-aspartate, as are the two other ionotropic receptors, the AMPA and kainate receptors. These non-NMDA receptors, permeable to monovalent cations such as Na^+ and K^+, mediate most of the 'fast' excitatory neurotransmission that occurs during normal brain activity. In contrast, NMDA receptors activate more slowly than non-NMDA receptors and exhibit slow desensitization to long pulses of exogenously applied glutamate. Consequently, NMDA receptor activation evokes an excitatory postsynaptic current (EPSC) which displays a slow rise time and a slow decay. These biophysical properties underlie the important role of NMDA receptors in synaptic plasticity events such as long-term potentiation.

Furthermore, the long duration of a NMDA receptor-mediated EPSC permits summation of subsequent EPSCs and can promote an increase in excitability. Another unusual feature of the NMDA receptor-operated channel is that, as well as passing monovalent cations, it possesses a relatively high permeability to Ca^{2+} ions. As a consequence, NMDA receptor activation results in a significant increase in $[Ca^{2+}]_i$ with repeated receptor stimulation, a feature which appears to be of importance in the established role of NMDA receptor activation in the genesis of both neurodegenerative and epileptiform phenomena (*e.g.* Dingledine *et al.*, 1986; Stasheff *et al.*, 1989; Rogawski, 1992; Muir and Lees, 1995; Rothman and Olney, 1995).

The NMDA receptor-operated channel is a complex multimeric protein containing numerous modulatory and other sites in addition to the agonist binding site. The former include glycine, proton, polyamine, magnesium and phencyclidine (PCP) binding sites. The glycine site is strychnine-insensitive, binding glycine which acts as a co-agonist with glutamate (the endogenous transmitter), such that in the absence of glycine the NMDA receptor-activated current is greatly diminished (Johnson and Ascher, 1987; Greenamyre and Porter, 1994). Compounds acting as antagonists at the glycine binding site have been shown to possess anticonvulsant activity (see Rogawski, 1992). Current flow through the NMDA receptor-operated channel is also inhibited by a rise in $[H^+]_o$ (*i.e.* a decrease in pH_o; Tang *et al.*, 1990; Traynelis and Cull-Candy, 1990; Vyklicky *et al.*, 1990). Not only does a fall in pH_o inhibit epileptiform activity mediated by NMDA receptor activation, but also external alkalosis leads to the development of epileptiform activity which, in turn, is sensitive to NMDA receptor antagonists (*e.g.* Aram and Lodge, 1987; Church and McLennan, 1989). The polyamine site is situated on the extracellular side of the NMDA receptor molecule at a site distinct from the ion channel. The endogenous polyamines spermine and spermidine bind to this site, as do the

synthetic agents ifenprodil and eliprodil (Legendre and Westbrook, 1991; Williams *et al.*, 1991; Rogawski, 1992; Muir and Lees, 1995). Both ifenprodil and eliprodil have demonstrated anticonvulsant and neuroprotective qualities, although whether this is due solely to an action at the polyamine site is questionable, as both compounds have also demonstrated voltage-activated Ca^{2+} channel blocking activity (Biton *et al.*, 1994; Church *et al.*, 1994b; Biton *et al.*, 1995). In addition, intracerebroventricular administration of spermine and spermidine can have proconvulsant effects (Singh *et al.*, 1991) although it remains unclear if this effect is related solely to a potentiation of NMDA receptor-mediated events by the polyamines.

The characteristic of the NMDA receptor which influences its function to perhaps the greatest extent is the voltage dependent magnesium (Mg^{2+}) ion channel block. In the absence of extracellular Mg^{2+}, the whole-cell current evoked by NMDA varies approximately linearly with the membrane potential. In the presence of Mg^{2+}, however, a reduction in current flow is observed at potentials more negative than about -30 mV (Nowak *et al.*, 1984). Consequently, current flow through the NMDA receptor-operated channel occurs only after the membrane is depolarized sufficiently to alleviate the Mg^{2+} block of the channel. This Mg^{2+} block plays a critical role in controlling the excitability of neurons which possess NMDA receptors, as demonstrated by the development of epileptiform activity in both human and animal neural tissue bathed in low Mg^{2+} solutions (Anderson *et al.*, 1986; Avoli *et al.*, 1987; Mody *et al.*, 1987; Traub *et al.*, 1994; see below). Interestingly, the administration of Mg^{2+} has been used for many years as standard therapy for seizures associated with preeclampsia (Muir and Lees, 1995).

The last modulatory site to be discussed is the phencyclidine (PCP) binding site, also found within the channel pore of the NMDA receptor-ionophore complex. PCP was first developed in the 1950s as a general anesthetic agent, although its clinical use was soon abandoned due to adverse neurobehavioural side effects and it has since become a substance of abuse ("angel dust"). Not until more than two decades later was it discovered that PCP, along with other dissociative anesthetics such as ketamine and related compounds such as dizocilpine (MK-801), were acting as NMDA antagonists by blocking the NMDA receptor-operated channel (Anis *et al.*, 1983; Wong *et al.*, 1986). These compounds are uncompetitive NMDA receptor antagonists (*i.e.* exhibit use- or agonist-dependency) and bind to the PCP site to reduce ion flux through the channel pore (reviewed in Rogawski, 1992; Löscher and Schmidt, 1994; Muir and Lees, 1995).

The NMDA receptor-channel complex was first demonstrated to be involved in epileptiform activity by Croucher *et al.* (1982) who observed seizure-like discharges following the iontophoretic application of NMDA onto normal neurons in the hippocampus, striatum and neocortex. This group, amongst others throughout the 1980s, also established the anticonvulsant activity of competitive NMDA receptor antagonists such as 2-amino-5-phosphonovalerate (APV or AP5) and 2-amino-7-phosphonoheptanoate (APH or AP7). Subsequent to the identification of the anticonvulsant activity of competitive NMDA receptor antagonists, it was recognized that the previously observed anticonvulsant activity of the uncompetitive antagonists such as PCP, ketamine and MK-801 could also be explained by their interaction with the NMDA receptor (reviewed in Porter and Rogawski, 1992; Löscher and Schmidt, 1994).

Although uncompetitive antagonists with high affinity for the PCP binding site, such as MK-801, possessed potent anticonvulsant properties in animal seizure models (*e.g.* Wong *et al.*, 1986; see above), their administration in human clinical trials induced psychotomimetic effects such as confusion and hallucinations and their evaluation was promptly terminated (see Löscher, 1993). Competitive NMDA receptor antagonists seem to possess a more favourable therapeutic index than their uncompetitive counterparts, exhibiting in particular a lower likelihood of inducing PCP-like psychotomimetic effects. Competitive NMDA receptor antagonists do, however, induce some motor side effects such as ataxia and motor weakness (Chapman and Meldrum, 1991; Porter and Rogawski, 1992). As such, their potential as therapeutically-useful anticonvulsant compounds is still under investigation, in both animal models and human clinical trials (Löscher and Schmidt, 1994). Nevertheless, competitive NMDA receptor antagonists suffer from some theoretical disadvantages as anticonvulsants compared to uncompetitive, channel-blocking agents. Thus, uncompetitive antagonists are not displaced from their site of action by the high concentrations of glutamate which can be observed during seizure activity; therefore, the higher the glutamate concentration, the more efficacious is the uncompetitive block. Furthermore, the use-dependent nature of their NMDA antagonism means that lower concentrations of uncompetitive NMDA antagonists than competitive NMDA antagonists may be associated with equally efficacious anticonvulsant activity (see Rogawski, 1993).

More recently, compounds have been identified which, in contrast to the high affinity PCP-site binding of uncompetitive NMDA antagonists such as MK-801, PCP and ketamine, act at the PCP site in a lower affinity manner (Löscher and Schmidt, 1994; Muir and Lees, 1995). Dextromethorphan (DXM), a widely used cough suppressant, represents an example of

such compounds. DXM is approximately fifteen times less potent an NMDA antagonist than PCP (Church *et al.*, 1989), with an IC_{50} value for reduction of NMDA-mediated responses lying in the range 0.55 μM (Netzer *et al.*, 1993) to ~ 6 μM (Church *et al.*, 1994c; Fletcher *et al.*, 1995). DXM possesses anticonvulsant activity in a number of animal models of epilepsy (*e.g.* Feeser *et al.*, 1988; Leander *et al.*, 1988; Tortella *et al.*, 1989) and is currently in human clinical trials (reviewed in Rogawski, 1993; Löscher and Schmidt, 1994) where dose escalation studies have revealed no clinical evidence of toxic side-effects (*e.g.* Fisher *et al.*, 1990; Albers *et al.*, 1991). The lack of PCP-like neurobehavioural side effects may be attributable to the higher off-rate constant for DXM (10 s^{-1}) compared to PCP or MK-801 (Church *et al.*, 1994c). Additional compounds with a relatively low affinity for the PCP binding site on the NMDA receptor-channel complex have now been identified and an examination of their anticonvulsant activities forms part of the present investigation.

Voltage-sensitive calcium channels

Ca^{2+} flux into neurons plays an essential role in the induction and propagation of convulsive activity (Dichter, 1989; Walden *et al.*, 1992; Löscher and Schmidt, 1994). This has been unequivocally demonstrated in animal and human studies which have shown seizure activity to be accompanied by an increase in intracellular Ca^{2+}, preceded by a decrease in the extracellular Ca^{2+} concentration (Heinemann and Hamon, 1986; DeSarro *et al.*, 1991; Löscher and Schmidt, 1994). The increase in intracellular Ca^{2+} is then thought to lead to the irreversible neuronal damage which accompanies excessive seizure activity (Dichter, 1989). In addition to Ca^{2+} influx through the NMDA receptor, voltage-sensitive calcium channels

(VSCCs) also contribute to the Ca^{2+} currents observed during epileptiform activity (Heinemann and Hamon, 1986; Miyakawa *et al.*, 1992; Löscher and Schmidt, 1994). Clinically, a number of the currently available antiepileptic drugs possess Ca^{2+} channel blocking activity as part of their spectrum of action (Rogawski and Porter, 1990; Dichter, 1993; see below).

Neuronal VSCCs, comprised of both low- and high-voltage activated (LVA and HVA) channels, have been further classified according to their kinetic behaviours and antagonist sensitivities, and each of the six subtypes of VSCCs contributes to a greater or lesser extent to 'epileptic' Ca^{2+} influx. T-type Ca^{2+} channels are LVA Ca^{2+} channels thought to mediate, at least in part, the intrinsic burst firing demonstrated by distinct neuronal populations in the CNS (Tsien *et al.*, 1988; White *et al.*, 1989; Spedding and Paoletti, 1992). A specific T-type channel antagonist does not exist; however ethosuximide, an efficacious therapeutic agent against absence seizures, acts at clinically-relevant concentrations to block T-type channels, suggesting a role for T-type VSCCs in these types of seizures (*e.g.* Coulter *et al.*, 1989). Their direct involvement in other seizure types, however, seems unlikely as ethosuximide is inactive against other epileptic disorders (Rogawski and Porter, 1990).

DHP sensitive L-type channels are HVA Ca^{2+} channels found predominantly on neuronal cell bodies and proximal dendrites (Gohil *et al.*, 1994; Kimura *et al.*, 1995). Their postsynaptic location precludes their involvement in neurotransmitter release (Barnes and Davies, 1988; O'Regan *et al.*, 1990; Horne and Kemp, 1991). They do however mediate postsynaptic Ca^{2+} influx once the postsynaptic cell has been depolarized sufficiently for their activation to occur (Miller, 1987; Meyer *et al.*, 1988; Tsien *et al.*, 1988). Their contribution to seizure activity however is questionable as DHP's, selective blockers of L-type channels, have

been found to show equivocal efficacy as anticonvulsant agents (Wauquier *et al.*, 1985; Dolin *et al.*, 1988; Morón *et al.*, 1990).

N-, P-, Q- and R-type channels are the most recently identified neuronal HVA VSCCs. The R-type channel lacks a specific antagonist and hence, the functions of this subtype have yet to be elucidated. It has not been be definitively associated with neurotransmitter release in any system, and its anatomical distribution is therefore likely limited to the somatic and/or proximal dendritic regions, similar to L-type Ca^{2+} channels (Miljanich and Ramachandran, 1995). In contrast, neurotransmitter release in the hippocampus is regulated by a combination of N- and P-/Q-type HVA Ca^{2+} channels which are insensitive to DHPs but selectively sensitive to various peptide toxins, such as the ω-conopeptides and ω-agapeptides. In addition to mediating presynaptic Ca^{2+}-dependent neurotransmitter release, N- and P-type Ca^{2+} channels have also been identified postsynaptically where they may contribute to depolarization induced postsynaptic Ca^{2+} entry (Burke *et al.*, 1993; Luebke *et al.*, 1993; Gaur *et al.*, 1994; Gohil *et al.*, 1994; Wheeler *et al.*, 1994a & 1994b; Kimura *et al.*, 1995; Malva *et al.*, 1995; Reuter, 1995).

The anatomical location of N-, P- and Q-types of HVA Ca^{2+} channels raises the possibility that Ca^{2+} channel blockers which act preferentially at these subtypes of VSCCs, thereby affecting both neurotransmitter release and postsynaptic Ca^{2+} influx, might demonstrate a more favourable anticonvulsant profile than compounds which act selectively on L-type channels (*i.e.* DHPs). Several lines of evidence seem to support this prospect, the first being that several clinically established anticonvulsant drugs such as phenytoin and the barbiturates have been shown to block DHP-insensitive Ca^{2+} influx and neurotransmitter release in rat cortical slices via blockade of N-type channels (Crowder and Bradford, 1987;

Rogawski, 1993; Löscher and Schmidt, 1994). Secondly, compounds such as adenosine and metabotropic glutamate receptor agonists, which act to limit neurotransmitter release via inhibition of presynaptic N-type Ca^{2+} channels, have demonstrated favourable anticonvulsant profiles of activity in numerous animal models (Prince and Stevens, 1992; Sheardown, 1992; Swartz and Bean, 1992; Yawo and Chuhma, 1993; Choi and Lovinger, 1996). Finally, organic Ca^{2+} channel blockers such as verapamil and flunarizine, which have activity against multiple subtypes of HVA Ca^{2+} channels (*cf* DHPs) (*e.g.* Ishibashi *et al.*, 1995), possess anticonvulsant properties both *in vivo* and *in vitro*, including activity against seizures which are resistant to DHPs (*e.g.* Bingmann and Speckmann, 1989; Aicardi and Schwartzkroin, 1990; DeSarro *et al.*, 1990; Czuczwar *et al.*, 1992; Pohl *et al.*, 1992).

Nevertheless, the role of Ca^{2+} channel blockade in the anticonvulsant activity of compounds such as verapamil and flunarizine remains controversial. For example, high concentrations of verapamil are required for antiepileptiform activity, leading to the suggestion that blockade of voltage-activated Na^{+} channels may in part be responsible for its anticonvulsant profile (Bingmann and Speckmann, 1989; Aicardi and Schwartzkroin, 1990). Similarly, flunarizine has a dual Na^{+}/Ca^{2+} channel blocking action and it remains unknown whether Na^{+} channel blockade or Ca^{2+} channel blockade might underlie its ability to reduce neurotransmitter release and act as an anticonvulsant agent (*e.g.* Tytgat *et al.*, 1991; Cousin *et al.*, 1993; Geer *et al.*, 1993). Newly identified compounds, similar to verapamil and flunarizine in that they possess blocking activity against multiple subtypes of HVA Ca^{2+} channels, but dissimilar in that they are known not to affect neuronal Na^{+} conductances, have been examined for anticonvulsant activity in the present study.

Sigma (σ) receptors

σ receptors are high-affinity binding sites which exist in the central nervous system as well as in many endocrine and immune tissues (reviewed by Deutsch *et al.*, 1988; Snyder and Largent, 1989; Walker *et al.*, 1990; Ferris *et al.*, 1991; Su, 1993). Their specific function in the CNS, however, has not yet been fully elucidated. A variety of structurally-dissimilar compounds, often referred to as σ receptor ligands, display high (nM) affinity for σ binding sites. Interestingly, some σ receptor ligands possess anticonvulsant activity and it has been suggested that this activity is mediated by the interaction of these compounds with high-affinity σ binding sites (see Walker *et al.*, 1990; Su, 1993). However many of these σ ligands also act as antagonists at NMDA receptors and/or VSCCs, albeit at μM concentrations, and the assignment of their anticonvulsant properties to an interaction with the high-affinity σ receptor has been rather presumptive and based primarily upon binding studies. As many of the compounds to be examined in this work demonstrate affinity for the σ receptor as well as for the NMDA receptor and/or VSCCs, the σ receptor and its role (if any) in the anticonvulsant activity of high-affinity σ ligands will be discussed further.

The high-affinity σ binding site was originally termed the σ-opioid receptor as it showed affinity for certain opioids such as pentazocine, cyclazocine and N-allylnormetazocine (SKF-10047; Largent *et al.*, 1984; Tam and Cook, 1984). However, the site was soon renamed the σ receptor when it was discovered that this site demonstrated poor binding affinity for naloxone, the archetypal opioid antagonist (see Su, 1982; Tam, 1983). Subsequently, the site was referred to as the σ/PCP site; however it has since been confirmed that the high-affinity σ binding site is distinct from the PCP binding site on the NMDA receptor-channel complex,

although many drugs do have affinity for both sites. Further classification of high-affinity σ binding sites ensued, and at least two subtypes of distinguishable sites have been identified, based on their affinities for certain σ ligands: the σ_1 site and the σ_2 site (Quirion *et al.*, 1992; see also Bowen *et al.*, 1989; Hellewell and Bowen, 1990). The σ_1 and σ_2 sites are distinguished from each other by their benzomorphan-preferring or nonbenzomorphan-preferring qualities, respectively. Another major difference between the σ_1 and σ_2 sites lies in their stereoselectivity, σ_1 sites preferring the dextrorotary isomers of σ ligands, while σ_2 sites preferentially bind levorotatory isomers (Su, 1991; Su, 1993). Although the biochemical and pharmacological characteristics of the high-affinity σ binding sites are becoming more clearly defined, the physiological functions of the various subtypes of high-affinity σ receptors still remain ill-defined and are currently under intense investigation.

That the σ receptor might be mediating the anticonvulsant activity of a number of compounds with high (nM) affinity for σ binding sites was first suggested by Musacchio *et al.* (1989), who demonstrated that ^{3}[H]dextromethorphan bound with high affinity to σ sites (see also Klein and Musacchio, 1989). It was suggested that the anticonvulsant activity of DXM, together with other non-opioid antitussives such as carbetapentane and caramiphen, was due to binding at high affinity DXM binding sites (which were later found to be equivalent to the σ_1 site; Zhou and Musacchio, 1991). Subsequently, the anticonvulsant activity of numerous σ ligands has been proposed to reflect their interaction, at nanomolar concentrations, with high-affinity σ binding sites (*e.g.* Aram *et al.*, 1989; Roth *et al.*, 1992; see also Tortella *et al.*, 1989). However, what seems to have been overlooked by many authors is the rather large discrepancy between the ligand concentrations used in σ site binding studies (nM) versus those required for

therapeutically useful anticonvulsant activity (μM). Recently, Fletcher *et al.* (1995) demonstrated that many compounds with high (nM) affinity for high-affinity σ binding sites also act, at μM concentrations, to antagonize NMDA receptor-mediated responses. Furthermore, many of the same compounds are also able to block multiple subtypes of HVA Ca^{2+} channels, again at μM concentrations (Church and Fletcher, 1995). In light of these findings, the suggestion was made that the anticonvulsant actions of σ receptor ligands arise as a result of their interaction with voltage-activated Ca^{2+} channels and/or NMDA receptors, rather than high-affinity σ binding sites. This discrepancy points to a need for an examination of the concentrations at which σ ligands demonstrate anticonvulsant activity, in order to decipher their likely sites(s) of action. This will be addressed in the present investigation using a series of σ site ligands.

Models of seizure activity

Many experimental models of seizure activity have been employed in the study of convulsive phenomena. Both *in vivo* and *in vitro* models have evolved, each having their own distinct advantages and disadvantages, but both contributing to our understanding of epilepsy and more recently to the development of anticonvulsant compounds.

In vivo models have as their greatest asset the fact that they represent what is occurring in the intact animal; they are however limited in their ability to decipher specific mechanisms underlying the convulsant activity and hence the way in which a drug is exerting its anticonvulsant action. Some *in vivo* models which have been developed include the genetically epilepsy prone rat, the photosensitive baboon, the seizure-prone gerbil and the

audiogenic mouse (reviewed in Engel, 1989). These species all exhibit seizure activity when stimulated by an external source such as light or sound. The pathophysiological mechanisms responsible for these seizures have yet to be demonstrated definitively, although these models do provide the opportunity to study mechanisms of neuronal excitability at the genetic level.

The other main class of *in vivo* models currently available is the kindling model of epilepsy. Unlike the genetic models, normal animals without a prior disposition to seizures are stimulated repeatedly using either chemical or electrical techniques, producing animals which are prone to seizures for an extended period of time following the stimulations (reviewed in Engel, 1989). Electrical kindling, for example, is induced by brief trains of subthreshold electrical stimuli over an extended period of time which eventually results in overt seizure activity. From this point onward, similar subthreshold stimuli which, prior to kindling, failed to produce any behavioural symptoms, will now induce seizure activity. Although the entire repertoire of alterations which underlie this model have not been entirely elucidated, there is definitive evidence for an increase in the number and/or sensitivity of NMDA receptors which accompanies the fully-kindled state (Mody and Heinemann, 1987; Martin *et al.*, 1992), further emphasizing the crucial role that these receptors seem to play in epileptogenesis.

In the past, many anticonvulsant agents have been discovered through empirical testing in the various animal models outlined above. These drugs were developed without knowledge of the mechanisms of epilepsy; indeed, in many cases, the mechanisms by which they exert their anticonvulsant effects are still being examined. Although this serendipitous approach has uncovered a number of drug therapies, it relies on the premise that drugs which are effective in animal models of seizure activity will be equally effective in humans. While this may be the case for some models and drugs, these *in vivo* animal models do not reveal any of the

mechanisms underlying seizures (Rogawski and Porter, 1990; Dichter, 1994). A more rational approach to drug development using *in vitro* models based on the specific cellular mechanisms which underlie seizures, should facilitate the development of new anticonvulsant compounds with more specific mode(s) of action and which, consequently, possess more acceptable toxicity profiles.

In vitro models of epilepsy have largely employed the hippocampal slice preparation, although studies utilizing the cortical slice are also encountered in the literature. The particular popularity of the hippocampal slice arises from its laminar structure and highly organized cellular layers. The slice preparation allows the researcher to control the microenvironment of the tissue, including such variables as the ionic makeup of the bathing fluid, the gas partial pressures, temperature, pH and the concentration of drug perfusing the slice. The slice preparation also offers the researcher visual control over the placement of both recording and stimulating electrodes, allowing access to specific neural circuitry within the slice (Teyler, 1980).

Epileptiform activity can be evoked in an hippocampal slice by manipulating the extracellular environment and/or by exposing the preparation to convulsant compounds such as penicillin, bicuculline, picrotoxin, tetraethylammonium (TEA) or 4-aminopyridine (4-AP) (see Dichter and Ayala, 1987; Avoli, 1988). Unfortunately, each of the chemical models of seizure activity affects, to a greater or lesser extent, inhibitory mechanisms; penicillin, bicuculline and picrotoxin, for example, inhibit $GABA_A$ receptor-mediated potentials whereas TEA and 4-AP, by blocking voltage-activated K^+ channels, enhance neurotransmitter release not only from excitatory but also inhibitory pathways (reviewed by Perreault and Avoli, 1989).

Altering the ionic composition of the extracellular fluid which perfuses the slices can also evoke seizure activity. The three most common ionic models are the high [K^+], the low [Ca^{2+}] and the low [Mg^{2+}] models. These models can be used singularly or in combination with each other. The high [K^+] model is based on the observed increase in extracellular [K^+] which accompanies seizure activity in the epileptic brain. The increased $[K^+]_o$ depolarizes the neuronal membranes due to alterations in the K^+ electrochemical gradient (Jefferys, 1990). The low [Ca^{2+}] model is also derived from ionic changes observed during seizure activity *in vivo*, namely the observed decrease in extracellular Ca^{2+} which precedes epileptic activity. Although the mechanisms involved in this model are not fully understood, a significant component is thought to arise from the reduction in surface charge screening due to the decreased $[Ca^{2+}]_o$ (Schwartzkroin, 1986; Dichter and Ayala, 1987). As complete removal of extracellular Ca^{2+} results in blockade of synaptic transmission, non-synaptic influences (see above), are also thought to be factors in the induction and propagation of epileptic activity in this model (Heinemann and Hamon, 1986; Yaari and Jensen, 1988).

Finally, the low Mg^{2+} or 'Mg^{2+}-free' model is a popular and well characterized model of epileptic phenomena. Epileptiform activity in this model can be attributed to the release of the NMDA receptor from its voltage dependent Mg^{2+} block, and increased membrane excitability through a decrease in surface charge screening (Mody *et al.*, 1987; Tancredi *et al.*, 1990). This model has a number of advantages over other *in vitro* models of epileptiform activity: firstly, no additional epileptogenic agent is needed; secondly, it is one of few models that does not interfere with inhibitory circuits (*e.g.* Tancredi *et al.*, 1990; Westerhoff *et al.*, 1995); thirdly, as the NMDA receptor has been proven to play an essential role in the initiation and propagation of seizure activity, activation of these receptors would seem to represent a fairly accurate

depiction of what may be occurring in the intact animal; and, finally, low Mg^{2+}-induced epileptiform discharges can be depressed by Ca^{2+} channel blockers, such as verapamil, which have activity at both DHP-sensitive and DHP-insensitive HVA Ca^{2+} channels (*e.g.* Pohl *et al.*, 1992). It is for these reasons that I have chosen to employ the Mg^{2+}-free model in assessing the anticonvulsant activity of my chosen compounds.

Summary and Objectives

Epilepsy is a neurological disorder afflicting a significant proportion of the population. Currently available anticonvulsant drugs may give rise to deleterious side effects, while a significant proportion of patients remain refractory to these currently available anticonvulsants. There is a pressing need for more tolerable and efficacious antiepileptic compounds.

The initiation and propagation of epileptic activity involves the activation of both NMDA receptors and VSCCs. While competitive and high affinity uncompetitive (PCP-like) NMDA receptor antagonists have demonstrated anticonvulsant potential, their neurobehavioural side effects may preclude their use in humans. However, low affinity uncompetitive NMDA receptor antagonists such as DXM seem not to possess such toxicity and may provide more viable anticonvulsant potential. Voltage sensitive Ca^{2+} channels also represent a potential target for antiepileptic drugs. Although results of studies examining the anticonvulsant activity of DHPs have been equivocal, antagonists which act at multiple Ca^{2+} channel subtypes seem to hold more promise and need to be investigated further.

Recently, a series of compounds have been identified which act either as low affinity uncompetitive NMDA antagonists, broad spectrum blockers of multiple subtypes of HVA Ca^{2+}

channels, or both. In light of the fact that the anticonvulsant efficacy of many currently available anticonvulsant drugs appears to reflect multiple sites of action, mixed NMDA/Ca^{2+} channel blockers may offer advantages over compounds which act only via a single mechanism to depress seizure activity. For example, therapeutic benefit related to these compounds might be additive whereas the toxicities resulting from interactions at each site might be different and non-additive.

The aim of this study is therefore to assess the anticonvulsant activity of a novel wide spectrum Ca^{2+} channel blocker (loperamide), mixed 'low affinity' NMDA/Ca^{2+} channel antagonists (caramiphen, carbetapentane, opipramol, ifenprodil, dextromethorphan and 1,3-di(2-tolyl)guanidine) and a high-affinity (PCP-like) uncompetitive NMDA receptor antagonist (dextrorphan). By comparing their anticonvulsant profiles of activity to their known NMDA receptor and Ca^{2+} channel blocking potencies, I will attempt to assess the relative contributions made by NMDA receptor antagonism and Ca^{2+} channel blockade to their anticonvulsant properties. I will also assess the location of action (pre- or postsynaptic) of some of the compounds which possess Ca^{2+} channel blocking activity, in order to determine whether blockade of neurotransmitter release might contribute to their anticonvulsant actions. The concentrations at which these compounds, many of which are ligands for the high-affinity σ binding site, are therapeutically effective will also reveal whether or not the σ receptor is involved in their anticonvulsant activities. As many of the test compounds have been used clinically for indications other than epilepsy and are known to be relatively free of negative side effects, the evaluation of their anticonvulsant actions may identify novel anticonvulsant compounds with a favourable separation of therapeutic and unwanted effects.

MATERIALS AND METHODS

Slice preparation

Experiments were performed using transverse hippocampal slices from male Wistar rats weighing 200 - 300g. Animals were decapitated under halothane anesthesia, followed by surgical removal of the brain which was then transferred to cold (0 - 4 °C) artificial cerebrospinal fluid (ACSF; see below). While bathed in the cold ACSF, hippocampi were dissected free and cut transversely into slices 400 μm thick using a McIlwain tissue chopper. Two to three slices were then transferred to an interface tissue chamber where they were placed on a nylon mesh at the interface between oxygenated, warmed ACSF (flow rate = 1.5 ml/min) and humidified gas (95% O_2, 5% CO_2). The temperature of the chamber was maintained between 34 - 35 °C and the slices were allowed to recover for 60 min before recordings were made. The remaining hippocampal slices were maintained in oxygenated (95% O_2, 5% CO_2) ACSF at room temperature for a maximum of 3 hours for use in subsequent experiments.

Solutions and drugs

The normal ACSF, in which the hippocampal slices were initially prepared and subsequently perfused during control stages of an experiment, contained (mM): NaCl, 125; KCl, 3.0; $NaHCO_3$, 24.0; NaH_2PO_4, 1.5; $MgSO_4$, 1.5; D-glucose, 10.0; $CaCl_2$, 2.0. The magnesium-free ACSF was prepared by omitting $MgSO_4$ from the normal ACSF. The ACSFs, which contained HCO_3^-, were continually bubbled with 95% O_2, 5% CO_2, the pH thus

stabilizing at 7.4. All drugs used in the study were prepared fresh from powder immediately prior to each experiment. Drugs were solubilized in distilled water with the exceptions of ifenprodil, 1,3-di(2-tolyl)guanidine (DTG) and loperamide which were dissolved in ethanol, methanol and dimethylsulfoxide (DMSO), respectively and these stock solutions were then added to ACSF to obtain the final working concentrations. The concentrations of ethanol, methanol or DMSO in the final working solutions never exceeded 0.1 %, which in control experiments had no effect on responses (data not shown). All drugs used in the study were obtained from Sigma Chemical Co. (St. Louis, MO), with the exceptions of ifenprodil, adenosine and DTG which were obtained from Research Biochemicals Inc. (Natick, MA).

Recording and stimulating techniques

Evoked responses recorded in the hippocampal CA1 region were generated by orthodromic stimulation of the Schaffer collateral-commissural (SC) pathway. A schematic diagram of the hippocampal slice preparation in Figure 1*A* shows the SC pathway and the placement of the stimulating and recording electrodes. A bipolar stimulating electrode was used, constructed from twisted 62 μm insulated Nichrome wire. The electrode tip was positioned on the slice under magnified visual control using a micromanipulator. Single or paired stimulations (square-wave pulses, 0.05 ms duration, 1 - 100 V intensity) were performed at a maximum frequency of 0.05 Hz (*i.e.* stimuli were applied at a maximum rate of one every 20 s).

Extracellular field potentials were recorded using low resistance (10 - 20 MΩ) micropipettes, constructed from thin-walled, fibre-containing glass (1 mm o.d. × 0.75 mm i.d.;

F. Haer and Co., Brunswick, ME). Electrodes were backfilled with 4M NaCl and coupled to the recording system via a 3M-KCl-filled half-cell in contact with a chlorided silver pellet. The bath indifferent electrode was a chlorided silver wire. The recording electrode was positioned in the CA1 pyramidal cell body layer for assessment of the anticonvulsant activity of the test compounds (IC_{50} establishment protocol). The input/output and paired pulse facilitation experiments also required a second recording electrode positioned in the dendritic layer of the CA1 pyramidal cells. Placement of the recording electrodes in the slice was under magnified visual guidance. The electrode(s) was then lowered into the slice using an inchworm controller (Burleigh Instruments, Inc., Fishers, NY) to a depth of 130 - 170 μm.

Evoked responses and spontaneous epileptiform activity were amplified (Axoclamp 2, Axon Instruments Inc., Foster City, CA) and displayed on an analog oscilloscope (Tektronix 5113, Tektronix Inc., Beaverton, OR) and/or a chart recorder (Gould 2200S, Gould Inc., Cleveland, OH). While the chart recorder traces accurately reflect the amplitude of slower potential changes, its frequency response is insufficient to allow it to follow fast spontaneous discharges, and these appear truncated in figures. Data were either stored on magnetic tape for later digitization and analysis (Racal Store 4DS, Racal Recorders Ltd., Hythe, UK) or were digitized on line via a 12 bit analog-to-digital interface (TL-1, Axon Instruments Inc.; digitization rate = 50 kHz) and stored on a hard-drive for later analysis. Voltage traces were filtered (DC - 10 kHz) before digitization with an analog Bessel filter (AI 2040, Axon Instruments Inc.). Data were analyzed either by hand (spontaneous activity) or using the pCLAMP software package (v. 6.0.3, Axon Instruments Inc.).

Experimental protocols

A. Assessment of anticonvulsant activity

Following transfer of an hippocampal slice to the interface chamber, the experimental protocol consisted of four or five periods characterized as follows:

Period I. Mg^{2+}-containing control solution:

Slices were perfused with normal Mg^{2+}-containing ACSF for 60 min, allowing for full recovery of the slice. Single orthodromic stimuli were then applied to the SC pathway over the range 10 - 100 V in 10 V increments and a stimulus intensity producing a single population spike of half maximal amplitude (measured in the CA1 pyramidal cell layer) was identified and then used for the duration of the experiment. To assess slice viability and the presence of inhibitory synaptic responses, paired orthodromic stimuli were then applied (using the stimulus intensity identified earlier) with interstimulus intervals (ISIs) ranging from 10 - 50 ms (in 10 ms increments). For the remaining periods of an experiment, the paired stimulations were always performed with an ISI of 50 ms. Any slice which demonstrated multiple population spikes in response to either the single or paired stimulations was discarded.

Period II. Mg^{2+}-free solution:

Following the 60 min period of perfusion with standard, Mg^{2+}-containing ACSF, slices were perfused with Mg^{2+}-free ACSF until spontaneous epileptiform bursts occurred at a consistent frequency and amplitude. This period of time varied from approximately 30 - 90 min. During this time, spontaneous activity was recorded continuously on a Gould pen recorder (see Fig. 4). At the end of this period, a series of spontaneous burst waveforms were recorded for later analysis. The slice was then stimulated with paired orthodromic stimuli (50

ms ISI) with the previously chosen stimulus intensity. From this period onward only paired stimuli were performed, as single stimuli did not consistently evoke multiple population spikes during perfusion with Mg^{2+}-free ACSF. Responses were displayed on an oscilloscope and stored for later analysis. The spontaneous and evoked epileptiform responses recorded at the end of the period of perfusion with Mg^{2+}-free ACSF period served as the control responses for the effect of the test compound.

Period III. Mg^{2+}-free solution + test compound:

Following the period of perfusion with Mg^{2+}-free ACSF, test compounds were added to Mg^{2+}-free ACSF and were perfused onto the slice for 60 min. Spontaneous activity was again continuously recorded throughout this period. After 60 min, representative spontaneous bursts (if any) were recorded and stored for later analysis. Paired orthodromic stimuli were then applied, using the same stimulation parameters which were employed in Period II, above. Responses were recorded and stored for later analysis.

Period IV. Mg^{2+}-free recovery:

Following the 60 min exposure to the test compound, slices were re-perfused with the Mg^{2+}-free solution used throughout period II (*i.e.* drug-free, Mg^{2+}-free ACSF). Recovery of epileptiform activity was monitored for up to 3 hours. Throughout the recovery period, spontaneous activity was continuously recorded on the chart recorder. Representative spontaneous bursts (if present) were recorded at 60 min time intervals for later analysis. Paired orthodromic stimuli were also applied at 60 min intervals using the parameters described in periods II and III. Responses were again stored for later analysis.

Period V. Mg^{2+}-containing recovery:

In some experiments (*e.g.* see Fig. 9), slices were re-perfused with standard, Mg^{2+}-containing ACSF following full recovery of epileptiform activity achieved during the Mg^{2+}-free recovery period (period IV). This period of reperfusion with Mg^{2+}-containing medium was conducted at least once (following exposure to one of the higher concentrations tested) for each compound examined. Paired orthodromic stimuli were again applied, at a time when spontaneous epileptiform activity had been abolished by the return to Mg^{2+}-containing medium. These recordings were employed to assess the viability of the slice and to determine whether the recovery of epileptiform activity following wash-out of a test compound reflected a true reversal of the anticonvulsant effect of a test compound or simply a deterioration in slice viability. With none of the compounds tested was a severe reduction in slice viability observed.

B. Input/output and paired-pulse facilitation experiments

These experiments were designed to assess the possible site(s) along the SC pathway where selected test compounds might be mediating their demonstrated anticonvulsant activity (determined in *A.* above). The following protocol was employed:

Period I. Mg^{2+}-containing control solution:

Following preparation, hippocampal slices were allowed to recover for 60 min in the interface chamber, perfused with normal, Mg^{2+}-containing ACSF. Slices were then tested for viability, as in *A.*, Period I above. Input/output functions (see below) were examined using single orthodromic stimuli over the intensity range 10 - 60 V in 10 V increments, with evoked

responses recorded simultaneously from both the CA1 pyramidal cell body and dendritic layers. For the paired pulse facilitation analysis, responses to paired stimuli were recorded in the CA1 pyramidal cell dendritic layer at ISIs of 20, 30 and 40 ms. Stimulus intensity was adjusted to elicit a first field excitatory postsynaptic potential (fEPSP) of half-maximal amplitude.

Period II. Mg^{2+}-containing ACSF + test compound:

After obtaining control responses in Mg^{2+}-containing ACSF slices were perfused with Mg^{2+}-containing, drug-containing ACSF, for 60 min. At the end of this period, input/output functions were recorded as described in period I. For paired pulse facilitation analysis, the stimulus intensity was readjusted to half-maximal intensity, and paired stimuli were again delivered at ISIs of 20, 30 and 40 ms.

Data analysis

A. Assessment of anticonvulsant activity

The anticonvulsant activity of the test compounds was analyzed by comparing responses evoked in Mg^{2+}-free medium prior to the application of the test compound with responses evoked at the end of a 60 min period of perfusion with drug-containing, Mg^{2+}-free medium. Prior to analysis of the evoked epileptiform responses, noise generated from electrical (primarily 60 Hz) interference during the recording was minimized by applying a boxcar smoothing function (pCLAMP; Axon Instruments, Inc.) to the digitized waveforms. Evoked waveforms acquired in pCLAMP were exported as ASCII files into a Microsoft Excel (v. 5.0) worksheet. These waveforms were then analyzed using a Visual Basic macro which

measured the total length of the line representing the evoked waveform (see Korn *et al.*, 1987; Apland and Cann, 1995). This method of data analysis was chosen as it takes into account changes in both the amplitude and number of population spikes in the recorded waveform. Measurements of the first and second field potential responses (FP1 and FP2, respectively) were performed independently and added together to produce a value which represented the total length of the response. Separate measurements of FP1 and FP2 were performed so as to avoid inclusion of stimulus artifacts in the measurements of the length of the line. For both FP1 and FP2, measurement began following the primary population spike (as this first spike represents normal synaptic transmission) and continued to a point where voltage had returned to baseline. However, in cases where the epileptiform activity continued throughout the entire recorded trace (*i.e.* 317 ms), the end of the FP1 waveform was set at the last aquisition point prior to the second stimulus artifact, while the end point of the FP2 waveform was the last aquisition point of the entire trace (*i.e.* the data point acquired at 317 ms following the start of the acquisition period). The time interval used to measure the length of the waveform evoked by the first stimulus (FP1) was of the same duration in Mg^{2+}-free solution as in drug-containing, Mg^{2+}-free solution, and likewise for FP2. Background noise was subtracted from the length of the waveforms evoked by both the first and second stimuli.

The percent inhibition of epileptiform activity by each concentration of each test compound was then calculated using Equation 1,

$$\% \text{ inhibition} = 100 - \frac{FP1_d + FP2_d}{FP1_c + FP2_c} \times 100 \qquad \text{(Equation 1)}$$

where FP1$_d$ and FP2$_d$ denote the length of the field potential responses in 'drug + Mg^{2+}-free ACSF' and FP1$_c$ and FP2$_c$ represent the length of the (control) field potential responses in 'Mg^{2+}-free, drug-free ACSF'. The effect of each concentration of each test compound was examined on a minimum of two (and usually > 4) different hippocampal slices, and the percent inhibition values are presented as mean ± s.e.m. To derive IC_{50} values (the concentration of test compound resulting in 50% inhibition of the control response evoked in Mg^{2+} -free medium), averaged data points were fitted to the logistic equation:

$$R = R_{max}\,[\text{concentration}^{n_H}/(\text{concentration}^{n_H} + IC_{50}{}^{n_H})] \qquad \text{(Equation 2)}$$

where R is the observed change at the test concentration, R_{max} is the maximum observed change, concentration refers to the test drug concentration, and n_H is the Hill coefficient.

B. Input/output and paired-pulse facilitation experiments

The input/output (I/O) functions were analyzed by comparing responses evoked in Mg^{2+}-containing medium prior to the application of a test compound with responses evoked at the end of a 60 min period of perfusion with drug-containing, Mg^{2+}-containing medium. Recordings were made from both the CA1 pyramidal cell body and dendritic layers. Cell body layer recordings were used to measure the slope of the fEPSP and the population spike amplitude, while the presynaptic volley amplitude was measured from the dendritic layer recordings. Raw data traces of the two types of responses with the parameters used in the analysis can be seen in Figure 2. I/O measurements were used to assess the following functions: A) stimulus voltage vs. population spike amplitude, which represents the overall I/O function of the SC pathway; B) stimulus voltage vs. presynaptic volley amplitude, which

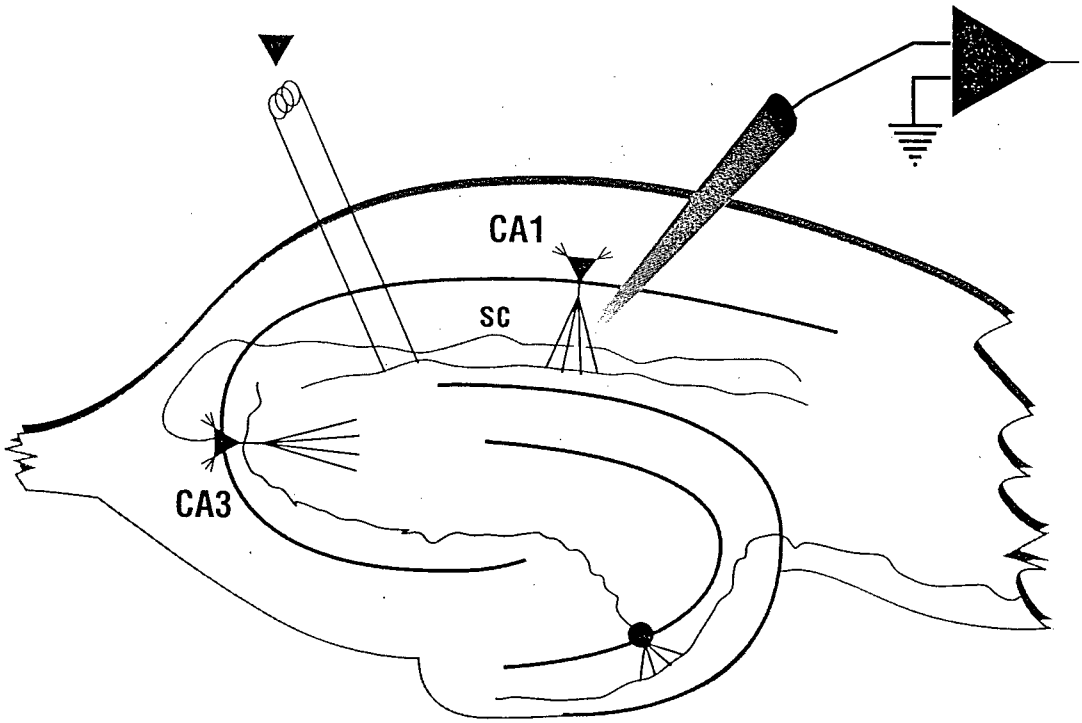
was employed to assess presynaptic fiber (Schaffer collateral) excitability; C) presynaptic volley amplitude vs. fEPSP slope, which represents the strength of CA3-CA1 synaptic transmission; D) fEPSP slope vs. population spike amplitude, which represents postsynaptic (CA1 pyramidal cell) excitability (Aitken, 1985; Balestrino *et al.*, 1986). I/O functions were assessed using single orthodromic stimuli, where each data point represents the average of three evoked responses, and four experiments were performed for each test compound at a concentration well above its previously established anticonvulsant IC_{50} value.

Under normal physiological conditions, if the SC pathway is stimulated twice in rapid succession (*e.g.* 20 - 70 ms ISI), the amplitude of the second field potential response (FP2) is potentiated when compared to the amplitude of the first field potential response (FP1), a form of synaptic plasticity known as paired pulse facilitation (PPF) (Alger and Teyler, 1976; Dunwiddie and Haas, 1985). The paired pulse ratio (FP2/FP1) changes only with manipulations that affect neurotransmitter release (Harris and Cotman, 1983; Dunwiddie and Haas, 1985; Simmons *et al.*, 1994). This protocol was therefore used to assess whether the anticonvulsant actions of certain test compounds were mediated by pre- or postsynaptic mechanisms. fEPSPs evoked using paired orthodromic stimuli were recorded from the CA1 pyramidal cell dendritic layer. For each interstimulus interval tested (*i.e.* 20, 30 and 40 ms) a PPF ratio (FP2/FP1) was calculated using the averaged amplitudes of three consecutively-evoked responses. Each test compound examined was tested in three or four hippocampal slices using a concentration well above its previously calculated anticonvulsant IC_{50} value.

Figure 1. Placement of the electrodes and intrinsic pathways in the hippocampal slice.

A. Schematic diagram of a rat transverse hippocampal slice, showing the placement of recording and stimulating electrodes. The stimulating electrode was placed on the Schaffer collateral-commissural (SC) pathway. For assessment of anticonvulsant activity, the recording electrode was positioned in the stratum pyramidale (cell body layer) of the CA1 region. For assessment of input/output functions and paired pulse facilitation, an additional recording electrode was placed in the stratum radiatum, as shown, to record dendritic field potentials. Diagram adjusted from Schuman and Kang (1996). ***B.*** Simplified local circuit diagram. Hippocampal afferent fibres in the SC pathway, in addition to synapsing directly on CA1 pyramidal cell dendrites, also send off collaterals which excite inhibitory (GABAergic) interneurons. These neurons then feed forward to make inhibitory synapses on CA1 pyramidal cell dendrites. Diagram modified from Lacaille *et al.* (1989).

A.


CA1
SC
CA3

B.

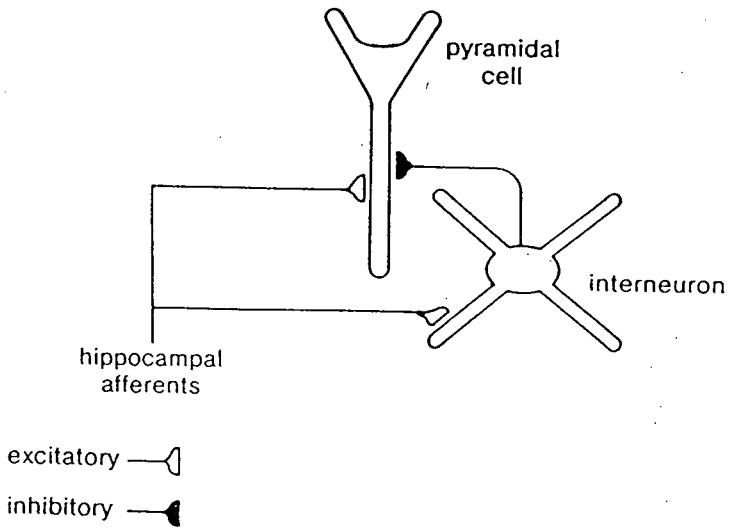
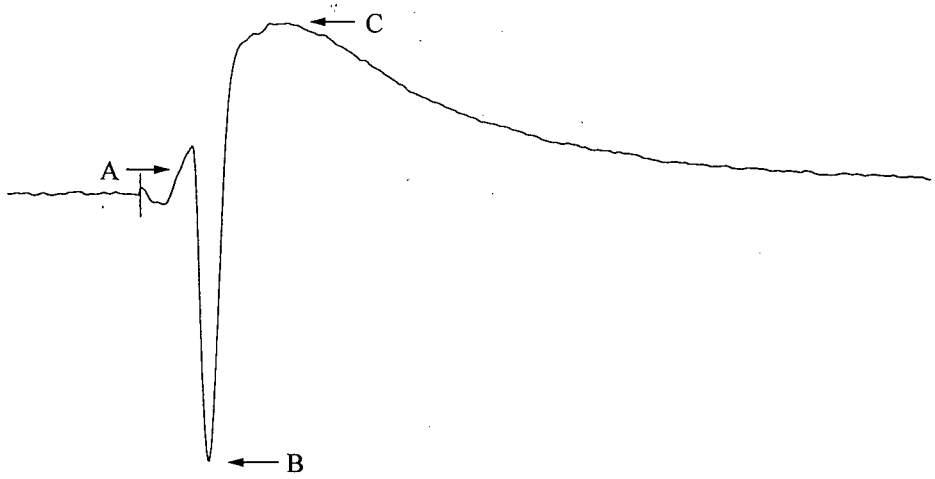

pyramidal cell
interneuron
hippocampal afferents
excitatory
inhibitory

Figure 2. Parameters employed for measurement of input/output (I/O) relationships and paired pulse ratios.

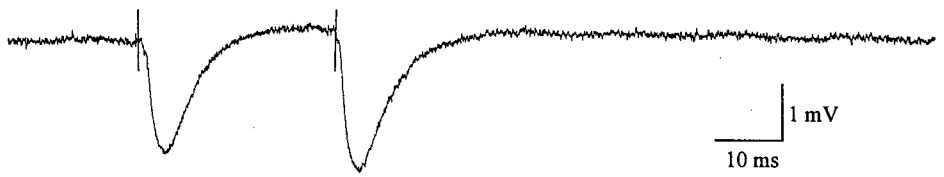
A. Typical waveform recorded from the CA1 pyramidal cell body layer in response to a single suprathreshold stimulation of the SC pathway. The slope of the fEPSP (mV/ms) was measured over a 0.5 ms segment taken from a midsection of line 'A'. Population spike amplitude (mV) was measured from the point halfway between 'C' and the peak of line 'A' to point 'B'. Stimulus artifacts, represented by the first biphasic deflection, were reduced in this and succeeding figures for clarity. ***B.*** Typical waveform recorded from the dendritic field of CA1 pyramidal neurons in response to single suprathreshold SC stimulation. Presynaptic volley amplitude (mV) was measured as the difference between 'D' and 'E'. ***C.*** Typical waveform recorded from the dendritic field of CA1 pyramidal neurons in response to paired subthreshold SC stimulation. Amplitude of fEPSPs was measured from baseline to the peak of the deflections. Scale bars shown in *B* also apply to *A*.

A.



B.

C.



RESULTS

Mg^{2+}-free epileptiform activity

Under normal physiological conditions (*i.e.* perfusion with Mg^{2+}-containing ACSF), paired stimulations of the SC pathway evoked a single population spike recorded in the CA1 stratum pyramidale following each stimulus (Fig. 3A). Population spikes represent the synchronous output of a population of neurons, and the amplitude of the population spike reflects the number of neurons participating in the output (Andersen *et al.*, 1971).

Epileptiform activity was induced by removal of Mg^{2+} ions from the perfusate. Under Mg^{2+}-free conditions, epileptiform potentials could be recorded from the CA1 pyramidal cell body layer in response to stimulation of the SC pathway (Fig. 3B). In most slices, paired stimuli evoked multiple population spikes following both the first and the second stimulus. However in a minority of instances (~ 15 %) paired stimuli failed to evoke multiple population spikes following either the first or the second stimulus (or both). Although the reasons for these failures to evoke epileptiform activity are unclear, possibilities include misplacement of the recording electrode and the presence of very powerful inhibitory influences. Slices in which paired stimuli failed to evoke < a total of 6 population spikes of at least 0.5 mV in amplitude were excluded from analysis.

Perfusion with Mg^{2+}-free medium was also associated with the development of spontaneous epileptiform activity which could be recorded from the CA1 pyramidal cell body layer (see Fig. 4). Such spontaneous epileptiform activity arises in the CA3 hippocampal region and propagates via the SC pathway to the CA1 region. Spontaneous epileptiform activity, which was present in 121/167 slices, developed gradually upon the introduction of the

Mg^{2+}-free ACSF, reaching a consistent amplitude and frequency after approximately 30 - 90 min, although recordings were not made until at least 45 min into the Mg^{2+}-free period of perfusion. The length of time required for the spontaneous activity to begin varied, the average time from the start of perfusion with Mg^{2+}-free medium until the start of spontaneous epileptiform activity being 21 ± 12 min (mean ± S.D.; n = 121). Once the spontaneous burst activity reached a plateau (*i.e.* steady-state) phase, the amplitude and frequency of the bursts also varied between slices, the amplitude from baseline to peak ranging from 0.5 - 4 mV, while the frequency ranged from 2 - 24 bursts/min. As noted in the Materials and Methods, spontaneous epileptiform activity was recorded during all phases of an experiment in which hippocampal slices were exposed to Mg^{2+}-free medium.

Although the effects of a test compound on spontaneous epileptiform activity were not assessed quantitatively (due to variability in the effects of a given concentration of a given test compound on either spontaneous burst frequency and/or amplitude between different hippocampal slices), a qualitative comparison was made between the effect of a given concentration of test compound on spontaneous epileptiform activity and its effect on epileptiform activity evoked by stimulation of the SC pathway. For example, if a certain concentration of a test compound consistently abolished spontaneous epileptiform activity while having only a minor effect on evoked epileptiform activity, the possibility exists that the compound may (for whatever reason) have a greater effect on the excitability of CA3 pyramidal neurons than on the excitability of CA1 pyramidal cells.

In some cases, slices demonstrated episodes of spreading depression, a phenomenon characterized by a large negative shift in membrane potential and accompanied by a drastic redistribution of extra- and intracellular ions; there is a large outflow of K^+ ions and an inflow

of Na^+, Cl^- and Ca^{2+} ions with a resultant influx of water, causing the neurons to swell (Mody *et al.*, 1987; Somjen *et al.*, 1992). During this time, spontaneous epileptiform activity was abolished and stimulation of the SC pathway failed to evoke any field responses. Both spontaneous and evoked epileptiform activity recovered within 10 - 15 min of the start of a period of spreading depression. Nevertheless, hippocampal slices which exhibited periods of spreading depression during perfusion with Mg^{2+}-free medium were not employed to assess quantitatively the anticonvulsant activity of test compounds. However, as this phenomenon has been shown to involve both NMDA receptor- and voltage-activated Ca^{2+} channel-mediated currents (Mody *et al.*, 1987; Jing *et al.*, 1991), it was interesting to note whether the test compounds were able to block the occurrence of spreading depression.

Anticonvulsant effects of the test compounds

i) Loperamide

Loperamide is an anti-diarrhoeal compound which recently has been shown to block both DHP-sensitive and -insensitive HVA Ca^{2+} channels in cultured hippocampal neurons (Church *et al.*, 1994a). In this regard, loperamide's spectrum of activity resembles that of verapamil, a 'broad-spectrum' blocker of multiple subtypes of HVA Ca^{2+} channels with established anticonvulsant activity (*e.g.* Bingmann and Speckmann, 1989; Aicardi and Schwartzkroin, 1990). However, in contrast to verapamil, loperamide does not block voltage-activated Na^+ channels and, unlike many of the compounds examined in this thesis, is only a very weak NMDA receptor antagonist (IC_{50} value for block of NMDA-receptor mediated whole cell current in voltage-clamped hippocampal neurons = 73 μM; Church *et al.*, 1994a).

Consequently, loperamide was employed as a compound representative of a class of agents which blocks multiple subtypes of HVA Ca^{2+} channels while having little activity at the NMDA receptor-channel complex.

Loperamide blocked epileptiform activity evoked by electrical stimulation of the SC pathway during perfusion with Mg^{2+}-free medium. The anticonvulsant effect was concentration-dependent, little suppression of evoked epileptiform activity being observed at concentrations $\leq$ 5 μM (Fig. 5). The anticonvulsant effect of loperamide was also fully reversible, recovery of epileptiform activity being observed within 60 min of the start of washout of low concentrations of the compound (Fig. 6). The IC_{50} value for inhibition of epileptiform activity evoked during perfusion with Mg^{2+}-free medium by loperamide was 28 μM (Fig. 7).

Loperamide was also able to inhibit the spontaneous epileptiform activity observed during perfusion with Mg^{2+}-free medium in a concentration-dependent manner (Fig. 8). A reduction in the amplitude of spontaneous bursts was observed at all concentrations of loperamide tested; a reduction in frequency, however, was only observed at concentrations > 5 μM. Interestingly, at the highest concentration tested (50 μM) loperamide failed to inhibit completely evoked epileptiform activity (see Figs. 5 and 7) whereas spontaneous epileptiform activity was abolished in 2/3 slices tested (see Fig. 8). Concentrations of loperamide > 50 μM could not be tested due to the limited solubility of the compound in aqueous solution.

Recurring episodes of spreading depression occurred in one hippocampal slice prior to exposure to loperamide. In this experiment, loperamide 10 μM blocked the occurrence of

spreading depression, which then re-appeared following 60 min of reperfusion with drug-free, Mg^{2+}-free medium (data not shown).

ii) Caramiphen, carbetapentane and opipramol

Caramiphen (CM) and carbetapentane (CBT) are antitussive agents which also possess anticonvulsant activity *in vitro* (Aram *et al.*, 1989; Apland and Braitman, 1990). Opipramol has been used clinically as an antidepressant agent and more recently has also been found to possess neuroprotective properties (Rao *et al.*, 1990). Also possessing both NMDA and HVA Ca^{2+} channel blocking properties, CM, CBT and opipramol were employed as compounds representative of mixed NMDA/Ca^{2+} channel blockers, but which exhibit a more potent action at HVA Ca^{2+} channels than at NMDA receptor channels (see Table 1).

CM blocked epileptiform activity evoked by stimulation of the SC pathway in a concentration-dependent manner. CM's inhibitory effects were fully reversible, as shown in Figure 9, where full recovery of evoked epileptiform activity was achieved approximately 120 min following drug washout. The IC_{50} value of CM for inhibition of Mg^{2+}-free evoked epileptiform activity was 46 μM (Fig. 10).

Spontaneous epileptiform activity was inhibited potently by CM with an observed decrease in both the amplitude and frequency of spontaneous bursts following addition of the compound to the Mg^{2+}-free perfusate (data not shown). Spontaneous activity was consistently abolished (13/14 slices) by even the lowest concentration (*i.e.* 20 μM) of CM tested (2/3 slices). CM did not show any preferential effects on spontaneous or evoked activity, inhibiting both parameters at each concentration tested. Spreading depression occurred in two

hippocampal slices prior to exposure to CM. In one slice, CM (100 μM) blocked irreversibly any further episodes of spreading depression, while in the other, 200 μM CM completely abolished all synaptic activity, including any spreading depression.

Epileptiform activity evoked by stimulation of the SC pathway during Mg^{2+}-free perfusion was also blocked by CBT in a concentration-dependent and reversible manner (Fig. 11). The IC_{50} value for CBT against epileptiform activity evoked by perfusion with Mg^{2+}-free medium was 38 μM, as shown in Figure 12.

Spontaneous epileptiform bursting was also attenuated by CBT; in 6/15 slices in which spontaneous activity was present, CBT (≥ 50 μM) abolished all spontaneous activity. At all concentrations above 20 μM, a decrease in both the amplitude and frequency of spontaneous bursts was observed, whereas perfusion with 20 μM CBT caused a decrease in burst amplitude accompanied by an increase in the frequency of bursts (in 3/6 slices which demonstrated spontaneous activity). Recovery of the spontaneous epileptiform activity was seen in 1/3 slices tested with concentrations of CBT ranging from 50 - 200 μM (data not shown). No differential effects on spontaneous or evoked activity were observed with CBT. Episodes of spreading depression occurred in 4 slices throughout testing of CBT. In two slices, CBT (50 μM) eliminated the spreading depression, while CBT (50 and 100 μM) had no effect in the remaining two slices.

Opipramol also effectively attenuated Mg^{2+}-free epileptiform activity evoked by stimulation of the SC pathway. The anticonvulsant activity was concentration-dependent (Fig. 13) and reversible, with recovery occurring after 60 min of reperfusion with drug-free, Mg^{2+}-

free medium following exposure to 50 μM opipramol (Fig. 14). The IC_{50} value for the anticonvulsant activity of opipramol was 52 μM, as shown in Figure 15.

Spontaneous epileptiform bursting observed in Mg^{2+}-free medium was also inhibited by opipramol. At high concentrations (*i.e.* 100 and 200 μM), both the frequency and amplitude of the spontaneous epileptiform bursts were decreased, with complete suppression of spontaneous activity occurring in 4/6 hippocampal slices. Lower concentrations (*i.e.* 20 and 50 μM) also decreased the amplitude and/or frequency of spontaneous bursts. The anticonvulsant effect of opipramol against spontaneous bursting was reversible, as shown in Figure 16. Similar to CM and CBT, at a given concentration, opipramol always inhibited both spontaneous and evoked epileptiform activity. Episodes of spreading depression did not occur in any of the hippocampal slices used throughout the investigations with opipramol.

iii) Ifenprodil, Dextromethorphan and 1,3-di(2-tolyl)guanidine

This group of agents possess nanomolar affinities for high-affinity sigma binding sites, in addition to their NMDA receptor channel blocking actions which are observed at micromolar concentrations. Recently, antagonism of HVA Ca^{2+} channels (also observed at micromolar concentrations) has also been identified as part of their spectrum of activity (Church and Fletcher, 1995). Ifenprodil, dextromethorphan (DXM) and 1,3-di(2-tolyl)guanidine (DTG) were therefore tested as compounds representative of low-affinity NMDA receptor antagonists with some novel HVA Ca^{2+} channel blocking activity (*cf* CM, CBT and opipramol which are more potent Ca^{2+} channel blockers than NMDA antagonists).

Interestingly, these compounds have demonstrated promising neuroprotective properties in recent studies (Pontecorvo *et al.*, 1991).

Evoked epileptiform activity induced by electrical stimulation of the SC pathway in Mg^{2+}-free medium was blocked by ifenprodil in a concentration-dependent manner (Fig. 17). The IC_{50} value for inhibition of evoked epileptiform activity in Mg^{2+}-free medium was 6 μM (Fig. 18). Although in the majority of cases (9/21 slices) ifenprodil demonstrated typical anticonvulsant effects, the evoked responses did vary quite markedly between the remaining slices. For example, in 4 of the 21 slices tested, perfusions with 10 or 20 μM ifenprodil actually resulted in proconvulsant effects, with the slice recovering back to its pre-drug activity level (*i.e.* less hyperexcitable) following reperfusion with drug-free, Mg^{2+}-free ACSF (data not shown). In addition, in 8 of the 21 slices tested, ifenprodil (20 - 50 μM) diminished the evoked response down to one small (0.5 - 1.5 mV) population spike following each of the paired orthodromic stimuli. In these slices, epileptiform activity failed to recover even after a prolonged period of reperfusion with drug-free, Mg^{2+}-free ACSF. This type of response can be seen following the second stimulus in Figure 17*C*. In order to examine whether this type of response reflected activation of the NMDA receptor, thereby allowing the membrane potential to depolarize to 0 mV, the competitive NMDA receptor antagonist 2-amino-5-phosphonovalerate (APV; 50 μM) was added to the Mg^{2+}-free ACSF after 180 min. of drug-free, Mg^{2+}-free recovery. However, in two experiments APV failed to reverse the profound inhibitory effect of ifenprodil on the evoked responses, indicating that an alternate and unknown mechanism must be mediating this effect of the compound.

Spontaneous epileptiform activity which occurred during perfusion with Mg^{2+}-free medium was blocked by ifenprodil, again in a concentration-dependent manner (Fig. 19). Ifenprodil (20 and 50 μM) reduced both the frequency and amplitude of the spontaneous bursts, while lower concentrations (1, 5 and 10 μM) often caused a decrease in the amplitude but an increase in the frequency of the bursts. However, in contrast to the effect of ifenprodil sometimes observed on evoked epileptiform activity, no overt proconvulsant effects of the drug on spontaneous activity were observed. In fact, on those occasions when ifenprodil increased evoked epileptiform activity, spontaneous epileptiform bursts were decreased in amplitude, although the frequency of bursts was increased. Spontaneous activity was completely abolished in 8/10 slices tested with 20 or 50 μM ifenprodil (Fig. 19), but recovered in only 1/5 slices tested for reversibility of the spontaneous epileptiform response. The effects of ifenprodil on spreading depression, which occurred in five slices varied, with ifenprodil (1 - 20 μM) either blocking or not affecting the episodes of spreading depression.

Dextromethorphan (DXM) consistently reduced the epileptiform activity evoked by stimulation of the SC pathway during perfusion with Mg^{2+}-free ACSF. Recovery from exposure to DXM occurred in 9/11 slices tested, although recovery time was quite prolonged (up to 360 min) following exposure to the entire range of DXM concentrations tested (see Fig. 20). The IC_{50} value for inhibition of evoked epileptiform responses in Mg^{2+}-free medium by DXM was 10 μM (Fig. 21).

DXM decreased both the amplitude and frequency of Mg^{2+}-free induced spontaneous epileptiform bursts in all slices tested ($n = 15$). In 3 slices, perfusion with 50 or 100 μM DXM completely blocked spontaneous activity. Similar to the evoked epileptiform responses,

extended periods of reperfusion with drug-free, Mg^{2+}-free medium were required before recovery of spontaneous epileptiform activity was observed (Fig. 22). DXM showed no preferential effects on either spontaneous or evoked epileptiform activity. Episodes of spreading depression occurred in two slices, with DXM (100 μM) abolishing this phenomenon in one slice, while 10 μM DXM showed no effect on spreading depression in the other slice.

1,3-di(2-tolyl)guanidine (DTG) also exhibited anticonvulsant activity against epileptiform bursts evoked by electrical stimulation of the SC pathway. The anticonvulsant effects of DTG were concentration-dependent (Fig. 23) and reversible, with slices able to recover to pre-drug levels of epileptiform activity in as little as 60 min following the start of reperfusion with drug-free, Mg^{2+}-free medium (Fig. 24). The IC_{50} value for the inhibition of evoked epileptiform activity by DTG was 15 μM, as indicated in Figure 25.

It was interesting to note that in certain cases, perfusion with DTG, despite having an overall inhibitory effect, actually increased the amplitude of the first (and sometimes subsequent) population spikes following electrical stimulation (see Fig. 26). An increase in the amplitude of the initial population spike(s) following both paired stimuli was observed in 4/18 slices tested with DTG (10 and 20 μM), while an increase following solely the second stimulus was seen in an additional four hippocampal slices exposed to DTG (10 and 20 μM).

Spontaneous epileptiform bursting induced by perfusion with Mg^{2+}-free medium was effectively inhibited by DTG. DTG caused a concentration-dependent decrease in both the amplitude and frequency of the bursts (Fig. 27). Recovery of pre-drug epileptiform responses was quite consistent, with recovery occurring in 7/8 slices tested for reversibility following exposure to 10 - 100 μM DTG (see Fig. 28). DTG (≥ 20 μM) abolished the spontaneous

activity in 7/21 slices. DTG consistently inhibited both spontaneous and evoked epileptiform activity at any given test concentration. Episodes of spreading depression occurred in two slices during DTG testing, but DTG (10 and 20 μM) demonstrated no effect on this type of activity.

iv) Dextrorphan

Dextrorphan (DX) possesses a high affinity for the PCP site of the NMDA receptor-channel complex with no Ca^{2+} channel blocking actions. DX was therefore used as a 'control' compound representative of compounds which exhibit NMDA receptor antagonism alone, with no Ca^{2+} channel blocking actions.

Stimulation of the SC pathway evoked epileptiform responses in Mg^{2+}-free medium, which were effectively inhibited by DX in a concentration dependent fashion (Fig. 29). Recovery of epileptiform activity following exposure to the highest concentration of DX tested (20 μM) was observed in both hippocampal slices where reversal was attempted (data not shown). The IC_{50} value for DX's inhibition of evoked epileptiform activity was 2 μM, as shown in Figure 30.

Similar to DTG, perfusion with DX containing, Mg^{2+}-free medium was occasionally observed to cause an increase in the amplitude of the first (and sometimes subsequent) population spike(s) following electrical stimulation of the SC pathway (Fig. 31). Potentiation of the initial population spike(s) following both stimuli was observed in 3/18 slices exposed to DX (2 - 5 μM), while an increased initial population spike(s) following only the second stimulus was observed in another three slices exposed to DX (1 - 2 μM).

Perfusion with Mg^{2+}-free medium induced spontaneous epileptiform bursting which was inhibited by DX. The anticonvulsant effect was concentration-dependent and was accompanied by a reduction in both the amplitude and frequency of the spontaneous bursts (Fig. 32). Spontaneous epileptiform activity was completely abolished by 10 - 20 μM DX in 3/17 slices tested (*e.g.* Fig. 32). The anticonvulsant effect of DX against spontaneous epileptiform activity was also reversible, as was observed in two hippocampal slices exposed to the highest concentration of DX tested (20 μM). No preferential effects of DX were observed on either spontaneous or evoked epileptiform activity. Episodes of spreading depression were observed in two slices; however, DX (2 and 10 μM) failed to block this type of activity.

Input/output and paired-pulse facilitation experiments

These experiments were performed with the intention of exploring possible site(s) of action and mechanisms which might be contributing to the anticonvulsant effects of certain test compounds. The input/output (I/O) experiments were performed in order to examine at which point along the CA3-CA1 pathway agents might be acting. Paired pulse facilitation (PPF) experiments were then used to investigate whether blockade of presynaptic Ca^{2+} channels responsible for neurotransmitter release might be involved in their anticonvulsant actions.

Adenosine was used as a control compound, as it has been well established that its inhibitory (*i.e.* anticonvulsant) actions are mediated via A1 receptors, which act to reduce presynaptic Ca^{2+} influx through N-type Ca^{2+} channels (Yawo and Chuhma, 1993; Wu and Saggau, 1994a). Experiments (n = 3) were performed using 300 μM adenosine dissolved in

Mg^{2+}-containing (control) solution. A high concentration was required as there exists a large amount of adenosine deaminase in hippocampal slices, an enzyme which catabolizes adenosine and therefore needs to be saturated before adenosine's effects can be observed (Dunwiddie and Hoffer, 1980). Figure 33 depicts the results of a typical experiment, in which the effects of adenosine on the I/O functions of the CA3-CA1 pathway were analyzed. Graph A represents the overall I/O function of the entire CA3-CA1 pathway. Graphs B through D then examine the component I/O functions along the SC pathway, as described in the Materials and Methods section.

Graph *A* of Figure 33 shows adenosine's overall inhibitory effect on the CA3-CA1 pathway. At any given stimulus intensity, adenosine reduced the amplitude of the population spike recorded in the CA1 pyramidal cell body layer in response to a single stimulus delivered to the SC pathway. A plot of stimulus intensity versus presynaptic volley amplitude (Graph *B*) shows that, at any given stimulus intensity, adenosine had no effect on the prevolley amplitude recorded in the CA1 dendritic field. This indicates that adenosine did not alter the excitability of the Schaffer collateral fibres. Graph *C*, which plots prevolley amplitude versus fEPSP slope indicates that for a given prevolley amplitude, adenosine caused a reduction in the corresponding fEPSP slope recorded in the CA1 pyramidal cell layer. This I/O function, which represents synaptic transfer, indicates that adenosine inhibited synaptic transmission occurring at the synapse between the CA3 pyramidal cell terminals and CA1 pyramidal cell dendrites. Finally, Graph *D*, which plots the fEPSP slope versus the population spike amplitude, represents the postsynaptic (CA1 pyramidal cell) excitability. The apparent difference seen in Graph *D* is actually due to adenosine's preceding effect on synaptic

transmission (Graph *C*); Graph *D* does not show a downward shift in the ordinate adenosine values, as does Graph C, indicating that adenosine did not affect postsynaptic cell excitability.

Although I/O functions are effective in deciphering a compound's point of action along a synaptic pathway, they are not able to identify whether an observed effect on synaptic transfer is mediated via pre- or postsynaptic mechanisms. Therefore, PPF experiments were performed, where an observed increase in the PPF ratio (FP2/FP1) has been shown to reflect a compound's ability to inhibit neurotransmitter release (Mallart and Martin, 1968; Dunwiddie and Haas, 1985). In contrast, if a compound is acting postsynaptically, a change in the PPF ratio is not observed. As shown in Figure 34, adenosine caused an increase in the ratio between the second and first fEPSPs, verifying its presynaptic mode of action (Harris and Cotman, 1983; Dunwiddie and Haas, 1985).

The second compound tested was DX, which was used as a negative control. Due to the presence of Mg^{2+} in the medium, NMDA receptors would have been essentially blocked, with responses to single orthodromic stimuli being mediated almost exclusively by AMPA/kainate receptors. As DX is known to act selectively at the NMDA receptor, one might predict that DX would have no effect on the I/O functions. As shown in Figure 35, this was indeed the case, with DX having no effect on any I/O function along the CA3-CA1 pathway. Furthermore, as an NMDA receptor antagonist with little Ca^{2+} channel blocking activity, DX would not be expected to influence neurotransmitter release, and should therefore have no effect on the PPF ratio. This was indeed observed (Fig. 36).

Loperamide, ifenprodil and CM were then examined for their effects on both I/O functions and PPF ratios. Loperamide (<100 μM; $n = 4$) and ifenprodil (20 μM; $n = 4$), which

both possess broad spectrum Ca^{2+} channel blocking activity, unexpectedly showed no effects on both the I/O functions and PPF ratios, similar to the results observed with DX (data not shown). CM (100 μM; $n = 4$), however, did seem to show an inhibitory effect on the overall I/O function of the pathway (see Fig. 37, Graph *A*). In a manner similar to that observed with adenosine, this inhibitory effect resulted from an inhibition of synaptic transmission, as depicted in Graph *C* of Figure 37. However, CM's effect on the PPF ratio was in direct opposition to the results observed with adenosine. Tested on 4 slices, CM 100 μM caused a significant decrease in the FP2/FP1 ratio, with the amplitude of the second fEPSP diminished markedly relative to the first (Figure 38). This phenomenon is termed paired pulse inhibition (PPI).

Blockade of multiple subtypes of HVA Ca^{2+} channels by CM is known to exhibit use-dependency (Church and Fletcher, 1995). Therefore, it was postulated that the PPI observed may in fact have reflected a presynaptic inhibition of neurotransmitter release which, due to CM's use-dependency, was only observed following the second stimulus. If this were true, if a third stimulus was given one might then observe a PPF when the amplitude of the second and third fEPSPs were compared. However, when slices were stimulated three times in rapid succession (interstimulus interval 40 ms), CM (100 μM) failed to cause a PPF, with the amplitude of the third fEPSP diminished in similar fashion to the second fEPSP ($n = 3$). Slices ($n = 3$) were also exposed to a one minute tetanus stimulation (0.1 Hz) prior to the paired pulse stimuli; however CM (100 μM) again caused a PPI, as above.

Table 1. Potency of selected compounds as NMDA antagonists and as inhibitors of barium currents (I_{Ba}) (representative of HVA Ca^{2+} channel blockade) in cultured hippocampal neurons under whole-cell voltage-clamp, as compared to *in vitro* anticonvulsant potency established in the present study.

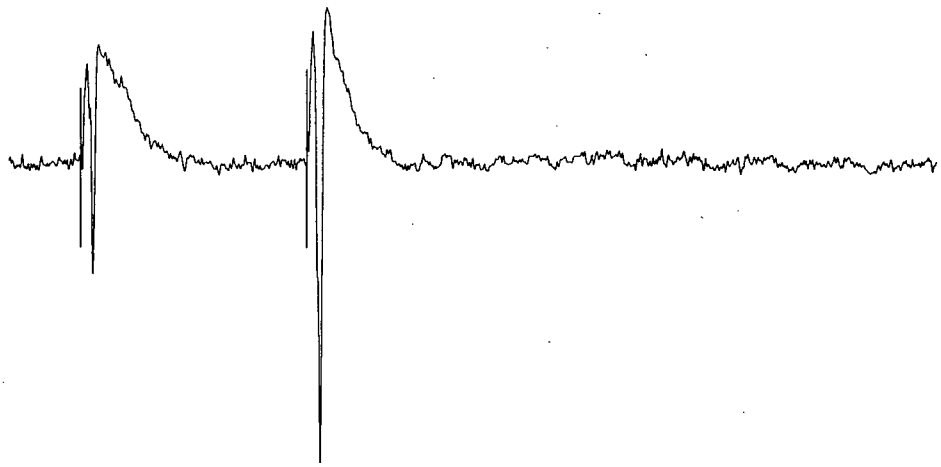
Data indicate mean ± s.e.m. of the IC_{50} values of the compounds, indicated to the left, for antagonism of NMDA-evoked currents in voltage-clamped neurons at V(h) = -60 mV (second column), for antagonism of whole-cell I_{Ba} in cultured hippocampal neurons (third column), and for anticonvulsant activity in hippocampal slices (fourth column). Data for the second and third columns taken from Fletcher *et al.* (1995) (NMDA receptor antagonism) and from Church and Fletcher (1995) (Ca^{2+} channel blockade), with the exception of dextrorphan (see Church *et al.*, 1989).

Compound	IC_{50} (μM) -- NMDA	IC_{50} (μM) -- I_{Ba}	IC_{50} (μM) -- slice preparation
Loperamide	73 ± 7	1.7 ± 0.3	28 ± 2
Caramiphen	110 ± 12	47 ± 3	46 ± 7
Carbetapentane	112 ± 13	40 ± 3	38 ± 5
Opipramol	96 ± 9	32 ± 4	52 ± 5
Dextromethorphan	1.8 ± 0.2	73 ± 10	10 ± 3
Ifenprodil	0.8 ± 0.2	18 ± 2	6.3 ± 0.6
Ditolylguanidine	37 ± 5	200 ± 20	15 ± 1
Dextrorphan	0.35	>100	1.5 ± 0.3

Figure 3. Representative traces of responses evoked by stimulation of the SC pathway in control and Mg^{2+}-free media.

A. Typical response, recorded in the CA1 pyramidal cell body layer, to paired stimuli (40 V; 50 ms interstimulus interval) in control (Mg^{2+}-containing) medium. Note the appearance of only a single population spike following either the first or the second stimulus. ***B.*** Following a 60 min period of perfusion with Mg^{2+}-free medium the same stimulus elicited an epileptiform response, consisting of multiple population spikes which followed both the first and second stimuli. Scale bars shown in *B* also apply to *A*.

A.



B.

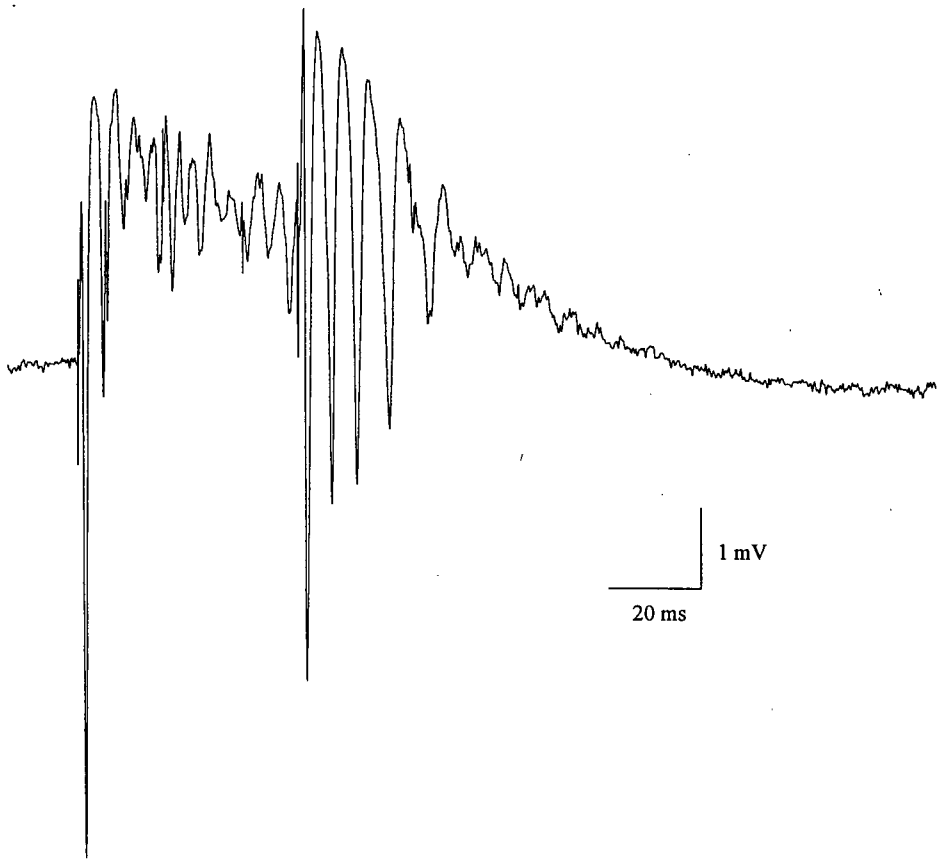
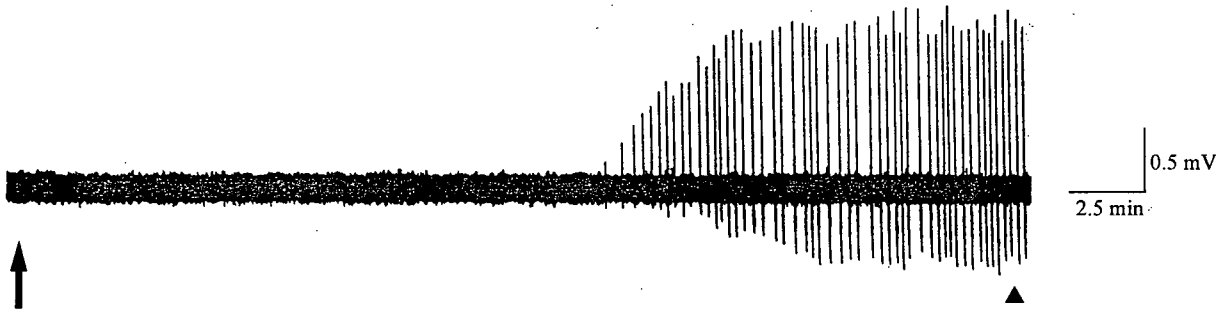


Figure 4. Representative traces of spontaneous epileptiform activity recorded during perfusion with Mg^{2+}-free media.

A. Continuous chart recording of spontaneous epileptiform activity recorded extracellularly in the CA1 stratum pyramidale. Spontaneous epileptiform activity developed following the introduction of Mg^{2+}-free medium (at the arrow) and gradually increased in amplitude and frequency until a relatively stable pattern of epileptiform activity emerged. ***B.*** Single spontaneous epileptiform burst captured at the symbol (▲) shown in *A* during perfusion with Mg^{2+}-free medium. Each single epileptiform burst corresponds to a single vertical line in the chart record. Note the expanded time base in *B*.

A.



B.

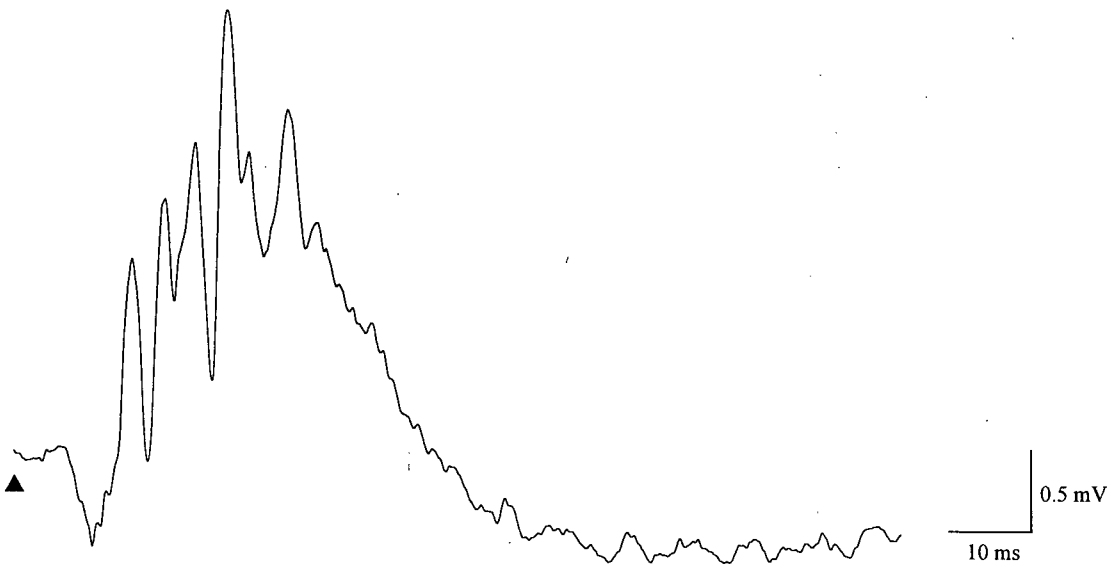
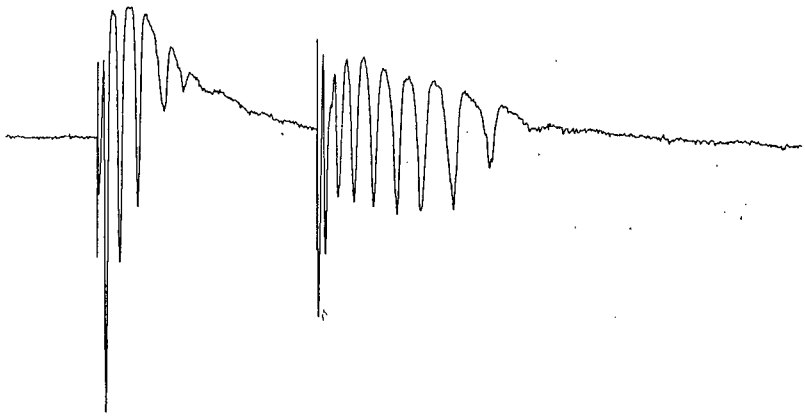


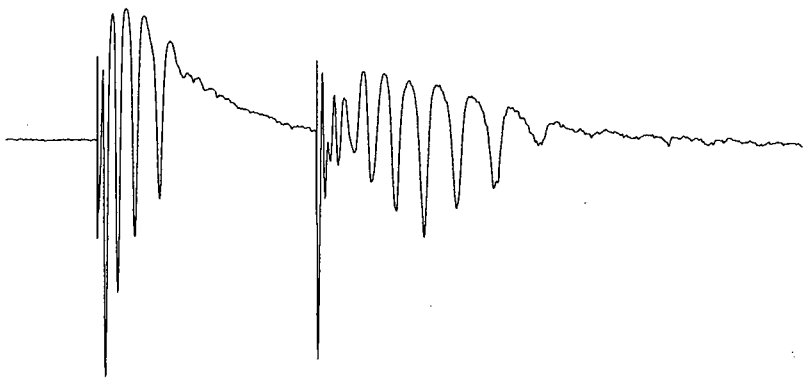
Figure 5. Concentration-dependent inhibition of evoked Mg^{2+}-free epileptiform activity by loperamide.

The SC pathway was stimulated using a double pulse paradigm (50 ms ISI). Extracellular field potentials were recorded from the CA1 pyramidal cell body layer. ***A.*** Following perfusion with Mg^{2+}-free ACSF for 60 min, multiple population spikes were evoked by paired orthodromic stimuli. ***B.*** Addition of 5 μM loperamide to the Mg^{2+}-free medium for 60 min failed to affect the evoked responses. ***C.*** Following the record shown in *B*, the concentration of loperamide in the Mg^{2+}-free perfusate was increased to 50 μM. Following 60 min of perfusion, 50 μM loperamide reduced the epileptiform response evoked by stimulation of the SC pathway. All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all traces.

A. Mg^{2+} - FREE, 60 min



B. Mg^{2+} - FREE + 5 μM LOPERAMIDE, 60 min



C. Mg^{2+} - FREE + 50 μM LOPERAMIDE, 60 min

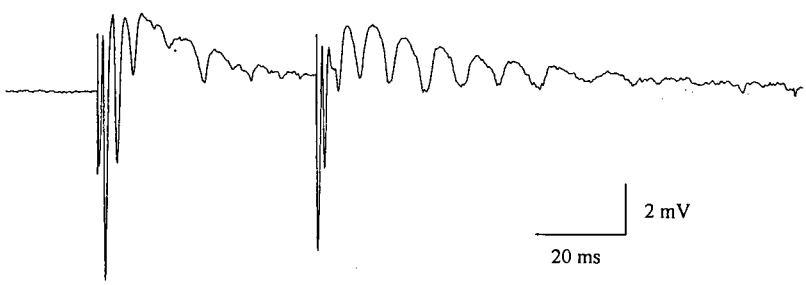
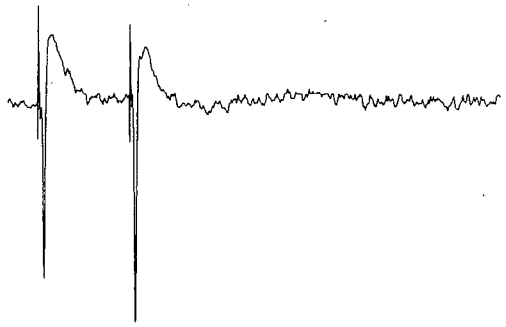


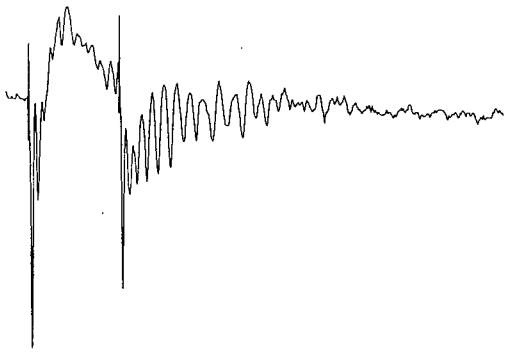
Figure 6. Recovery from the anticonvulsant effects of loperamide.

A. Evoked responses following 60 min perfusion with control (Mg^{2+}-containing) ACSF. Note the single population spike, recorded in the CA1 pyramidal cell body layer, following either the first or second stimulus. ***B.*** Paired stimuli delivered following a 60 min exposure to Mg^{2+}-free ACSF evoked epileptiform activity, particularly noticeable after the second stimulus. ***C.*** Epileptiform activity was attenuated following the addition of 10 μM loperamide to the Mg^{2+}-free perfusate. ***D.*** Recovery of the Mg^{2+}-free epileptiform response was observed 60 min following the start of reperfusion with drug-free, Mg^{2+}-free medium. All records were obtained from the same hippocampal slice. Scale bars shown in *D* apply to all traces.

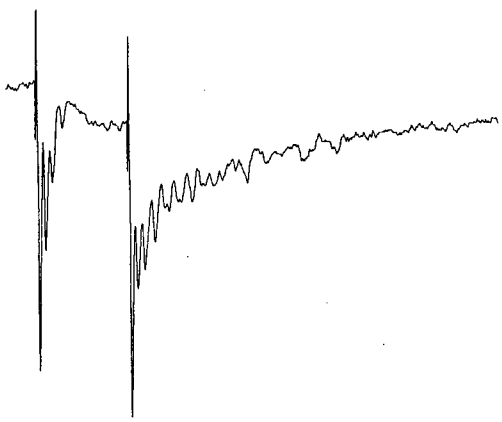
A. Mg^{2+} - CONTAINING CONTROL, 60 min



B. Mg^{2+} - FREE, 60 min



C. Mg^{2+} - FREE + 10 μM LOPERAMIDE, 60 min



D. Mg^{2+} - FREE RECOVERY, 60 min

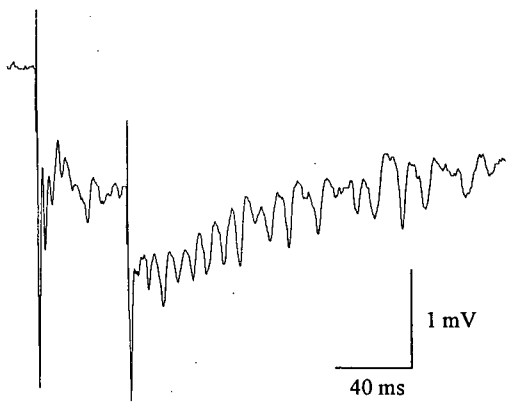


Figure 7. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by loperamide.

IC_{50} value for the inhibition of evoked epileptiform activity by loperamide was 28 μM, with a Hill coefficient of 1.74 and a Pearson product-moment correlation coefficient (r^2) of 0.94. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of loperamide in μM. Ordinate scale: % inhibition of evoked epileptiform activity. As detailed in the Materials and Methods, percent inhibition was calculated by comparing the 'length of the line' of the epileptiform response in Mg^{2+}-free medium to that of the response in drug-containing, Mg^{2+}-free medium. In this and succeeding log concentration-inhibition plots, data points which are not accompanied by error bars indicate that the error bars were smaller than the symbol.

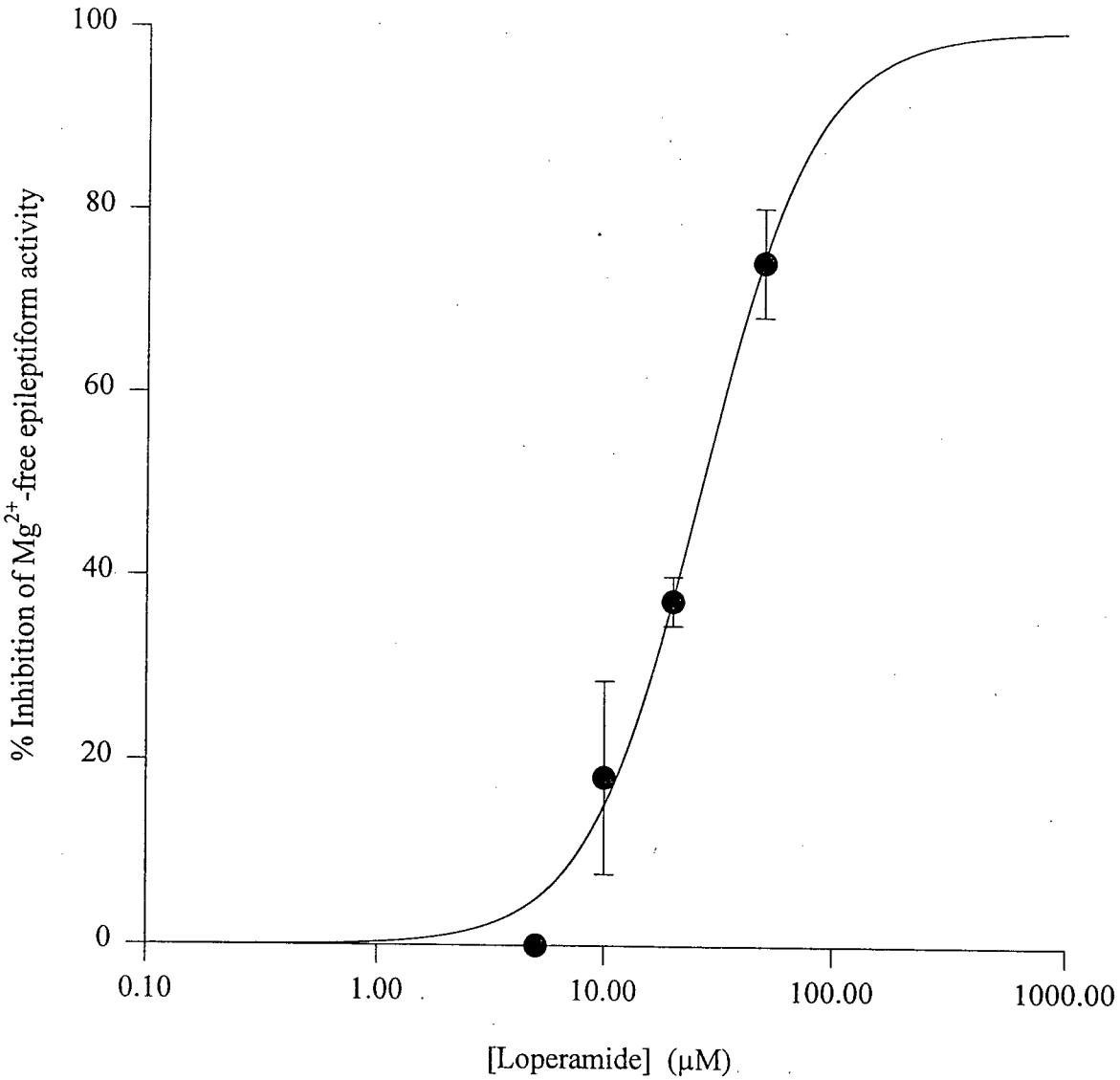
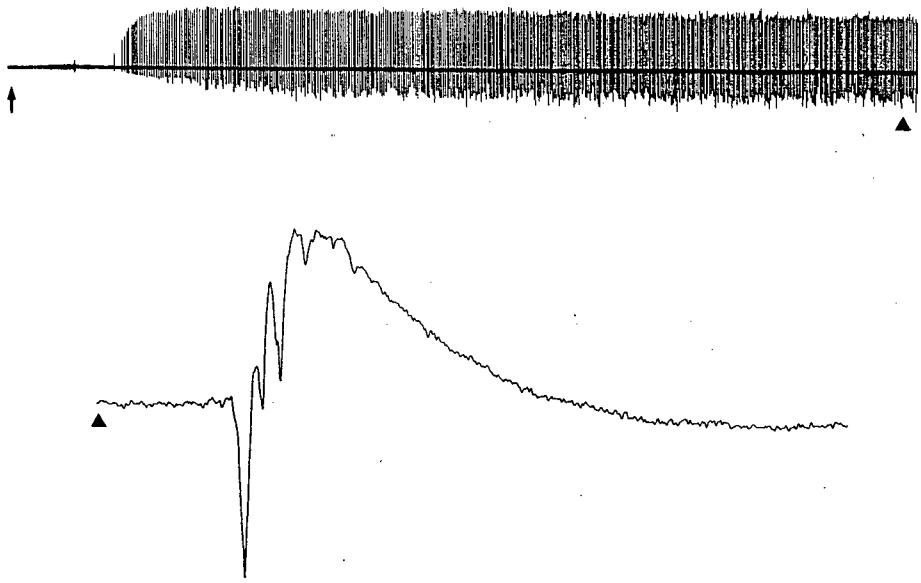

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
0.10
1.00
10.00
100.00
1000.00
[Loperamide] (μM)

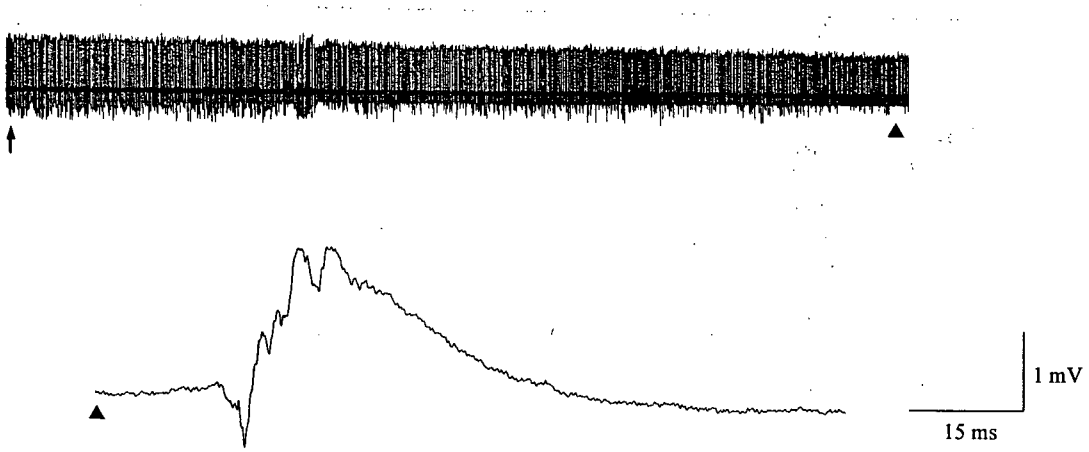
Figure 8. Concentration-dependent inhibition of spontaneous Mg^{2+}-free epileptiform activity by loperamide.

The lower traces in *A* and *B* represent single spontaneous bursts captured at the corresponding symbol (▲) in the chart recorder trace above. The absence of a representative single spontaneous burst in *C* indicates that spontaneous activity was abolished, as can be seen in the accompanying chart record. Arrows represent the initiation of perfusion with the media identified on the figure. ***A.*** Spontaneous epileptiform bursts developed following the introduction of Mg^{2+}-free ACSF and rapidly attained a stable amplitude and frequency. ***B.*** Following 60 min of perfusion with 5 μM loperamide, there was a slight decrease in the amplitude of the spontaneous bursts, as seen in the chart record and the single spontaneous burst shown. ***C.*** Exposure to 50 μM loperamide completely abolished spontaneous activity after a 50 min period of perfusion. All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *B*.

A. Mg^{2+} - FREE, 0 - 60 min



B. Mg^{2+} - FREE + 5 μM LOPERAMIDE, 0 - 60 min

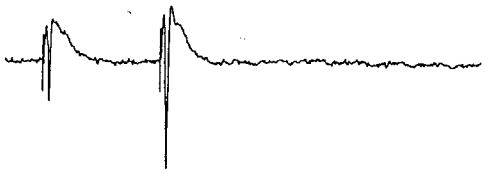


C. Mg^{2+} - FREE + 50 μM LOPERAMIDE, 0 - 60 min

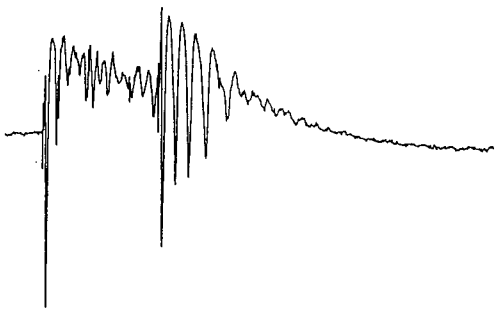
Figure 9. Reversible anticonvulsant effects of caramiphen (CM) against evoked Mg^{2+}-free epileptiform activity.

A. Following a 60 min period of perfusion with control (Mg^{2+}-containing) ACSF, single population spikes were evoked following a paired stimulation of the SC pathway. ***B.*** Epileptiform activity was evoked with paired stimuli following a 60 min period of perfusion with Mg^{2+}-free medium. ***C.*** Attenuation of evoked epileptiform activity after a 60 min exposure to 50 μM CM in Mg^{2+}-free ACSF. ***D.*** Epileptiform activity was re-established 60 min after the return to drug-free, Mg^{2+}-free perfusate. ***E.*** 120 min following drug washout, Mg^{2+}-free epileptiform activity had recovered almost fully back to the level seen in *B*. ***F.*** Epileptiform activity was arrested 30 min following the return to Mg^{2+}-containing ACSF. All records were obtained from the same hippocampal slice. Scale bars shown in *F* apply to all traces.

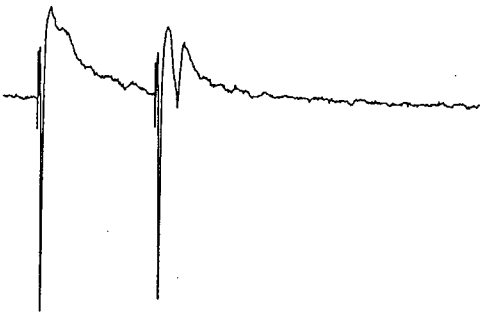
A. Mg^{2+} -CONTAINING CONTROL, 60 min



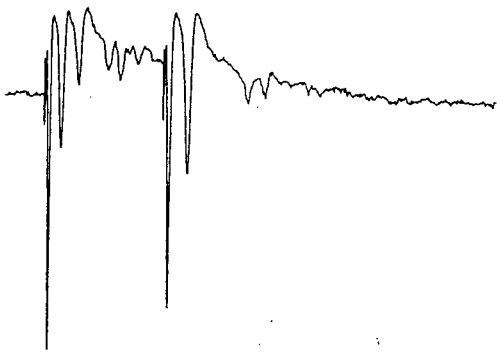
B. Mg^{2+} -FREE, 60 min



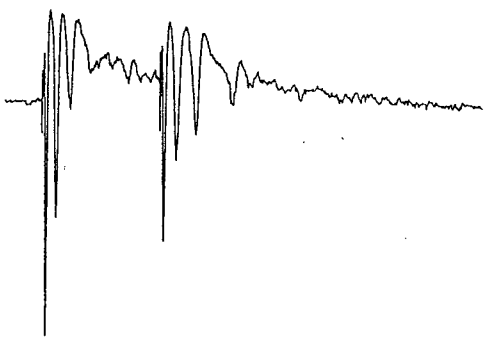
C. Mg^{2+} - FREE + 50 μM CARAMIPHEN, 60 min



D. Mg^{2+} - FREE RECOVERY, 60 min



E. Mg^{2+} - FREE RECOVERY, 120 min



F. Mg^{2+} - CONTAINING RECOVERY, 30 min

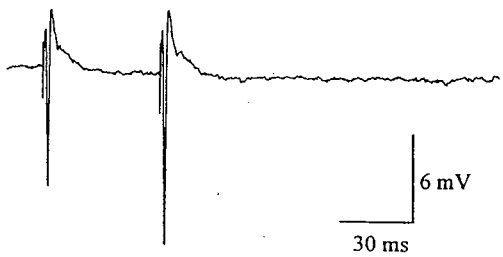


Figure 10. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by CM.

IC_{50} value for the inhibition of evoked epileptiform activity by CM was 46 μM, with a Hill coefficient of 1.93 and a r^2 value of 0.98. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of CM in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

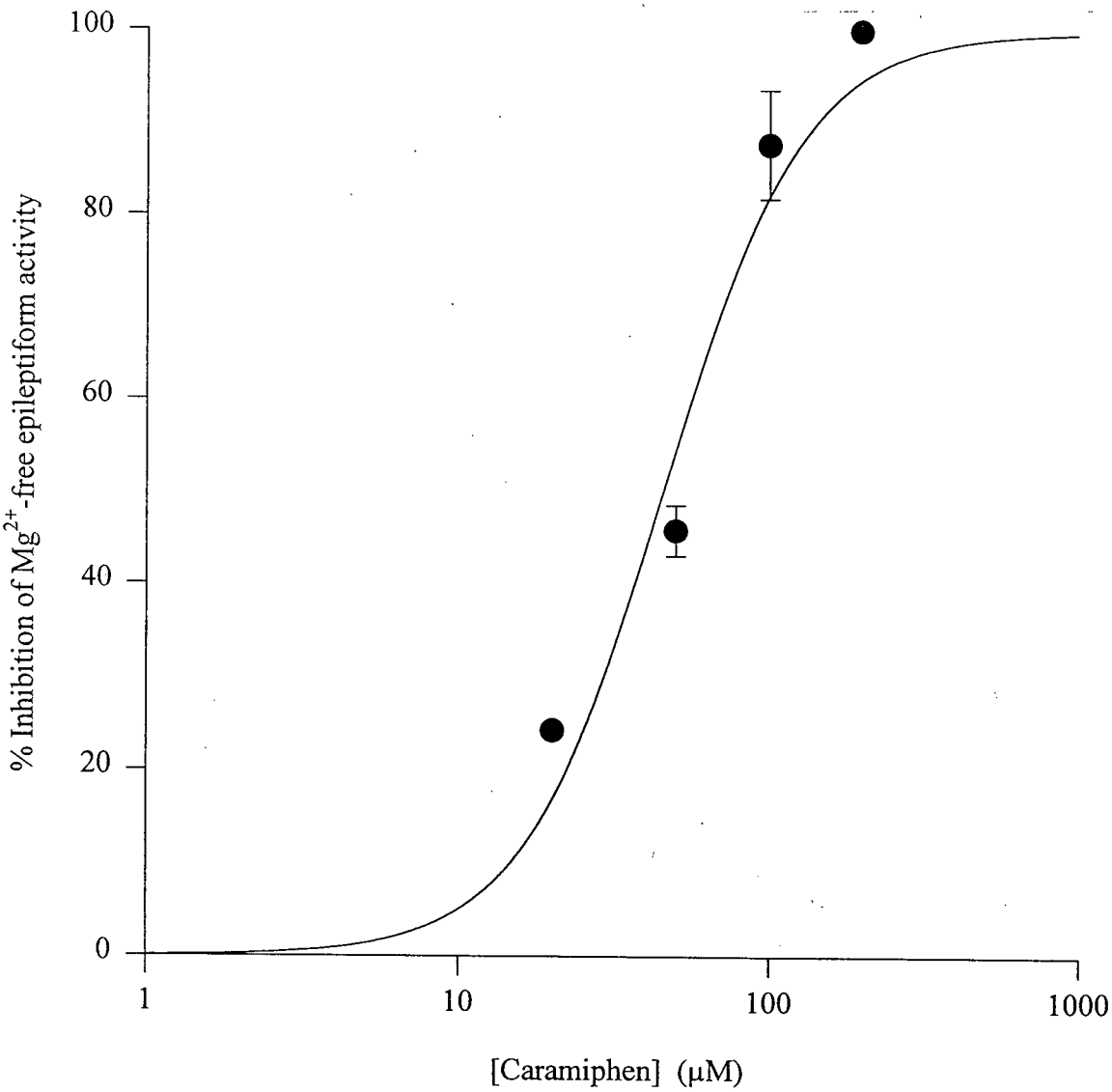
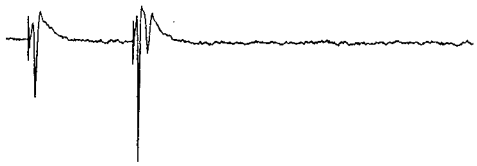

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
1
10
100
1000
[Caramiphen] (μM)

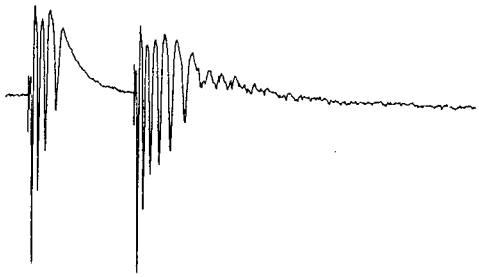
Figure 11. Carbetapentane (CBT) reversibly blocked Mg^{2+}-free evoked epileptiform activity.

A. Evoked response to paired stimuli following a 60 min period of perfusion with control (Mg^{2+}-containing) ACSF. ***B.*** Following a 60 min exposure to Mg^{2+}-free ACSF, the same paired stimuli evoked epileptiform activity. ***C.*** Exposure to 100 μM CBT for 60 min almost completely blocked the epileptiform activity seen in *B*. ***D.*** No change was seen in the evoked response 60 min after wash out of the drug. ***E.*** Mg^{2+}-free epileptiform activity began to recover 120 min following reperfusion with drug-free, Mg^{2+}-free ACSF. ***F.*** A more pronounced recovery of epileptiform activity was seen 180 min after drug washout. ***G.*** Reintroduction of Mg^{2+}-containing ACSF allowed the slice to recover almost fully to its control response, as in *A*. All records were obtained from the same hippocampal slice. Scale bars shown in *G* apply to all traces.

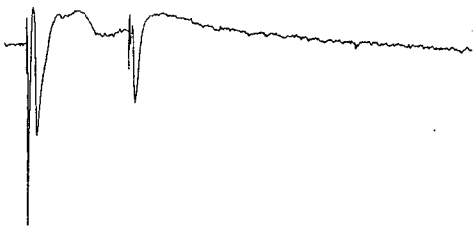
A. Mg^{2+} - CONTAINING CONTROL, 60 min



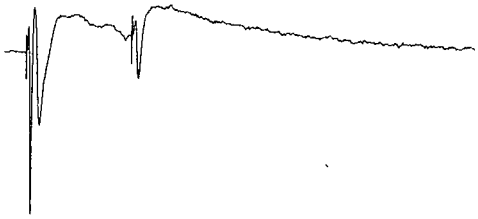
B. Mg^{2+} - FREE, 60 min



C. Mg^{2+} - FREE + 100 μM CBT, 60 min

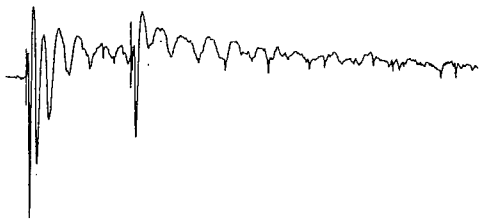


D. Mg^{2+} - FREE RECOVERY, 60 min



E. Mg^{2+} - FREE RECOVERY, 120 min

F. Mg^{2+} - FREE RECOVERY, 180 min



G. Mg^{2+} - CONTAINING RECOVERY, 30 min

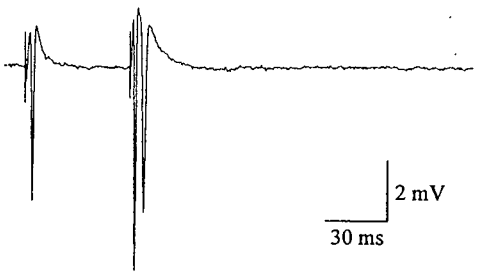


Figure 12. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by CBT.

IC_{50} value for the inhibition of evoked epileptiform activity by CBT was 38 μM, with a Hill coefficient of 2.09 and a r^2 value of 0.96. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of CBT in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

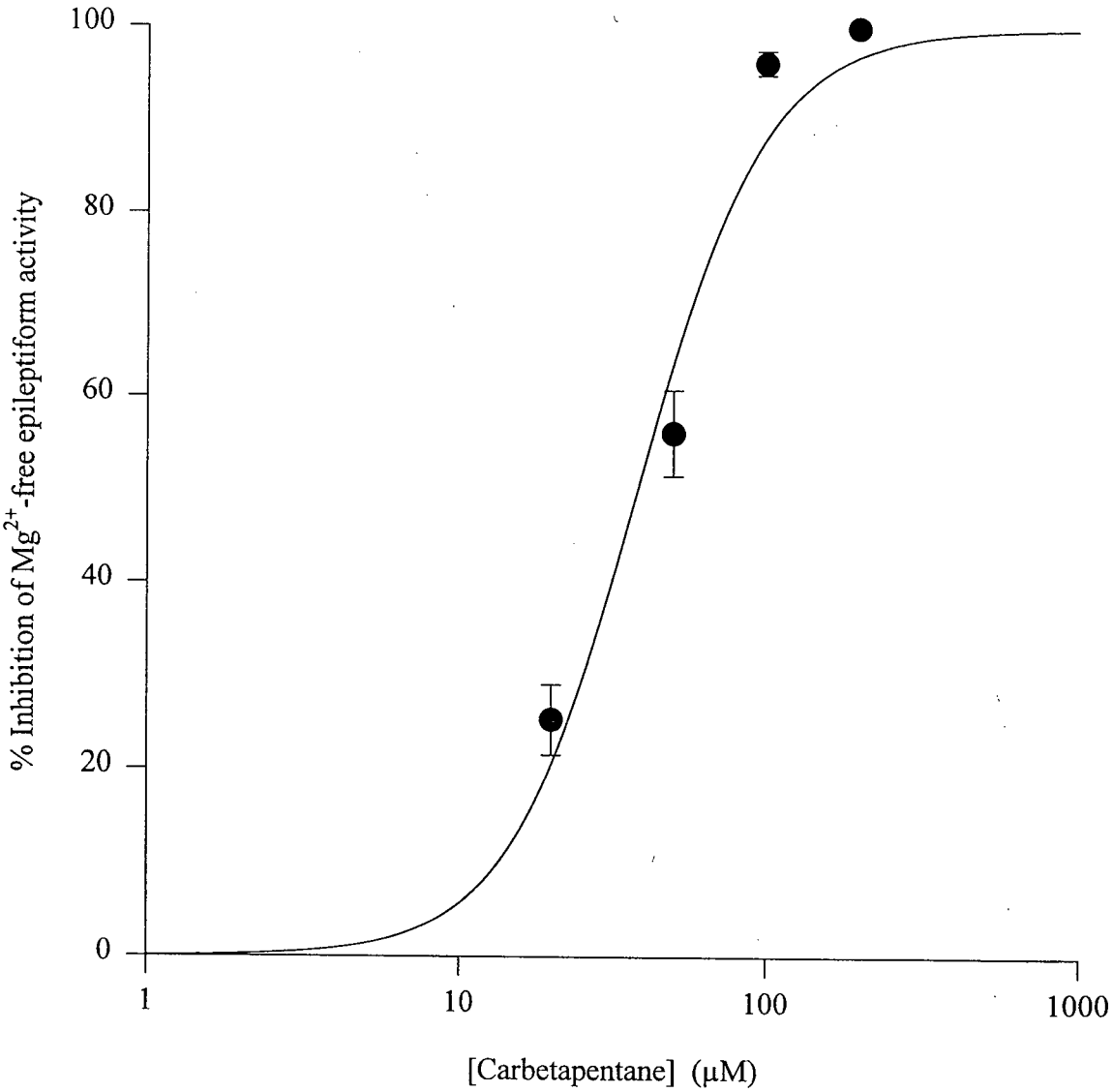
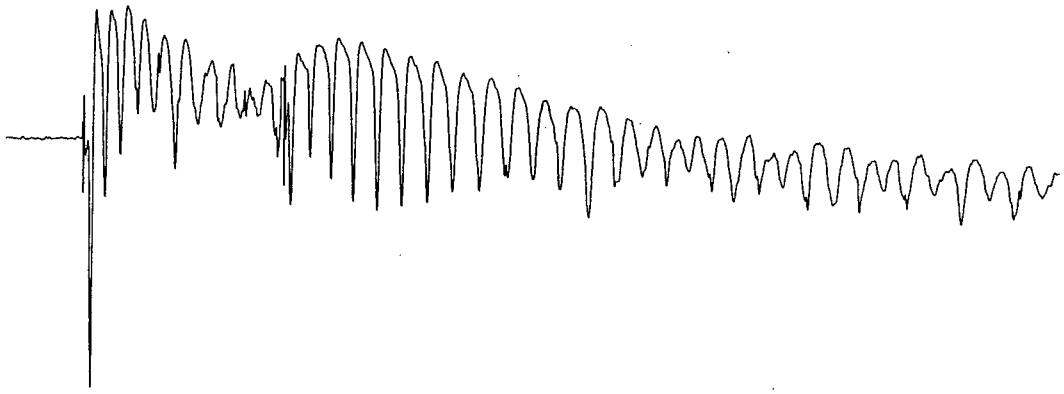

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
1
10
100
1000
[Carbetapentane] (μM)

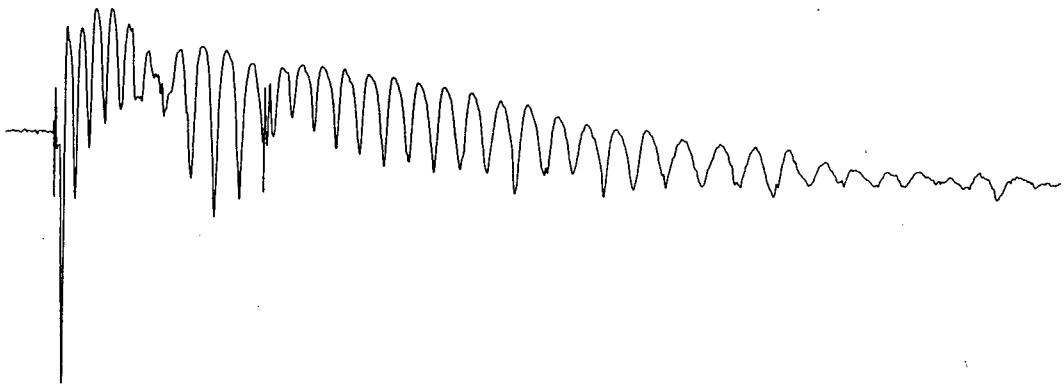
Figure 13. Opipramol inhibited Mg^{2+}-free evoked epileptiform activity in a concentration-dependent manner.

A. A 60 min exposure to Mg^{2+}-free ACSF induced epileptiform activity in response to paired orthodromic stimulation of the SC pathway. ***B.*** Following a 60 min exposure to 20 μM opipramol, the number and amplitude of the population spikes were slightly reduced. ***C.*** After the 60 min exposure to 20 μM opipramol shown in *B*, the slice was then perfused with 50 μM opipramol for 60 min. At the end of 60 min, the epileptiform activity was depressed significantly, with an almost complete inhibition of the epileptiform response following the second stimulus. All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all traces.

A. Mg^{2+} - FREE, 60 min



B. Mg^{2+} - FREE + 20 μM OPIPRAMOL, 60 min

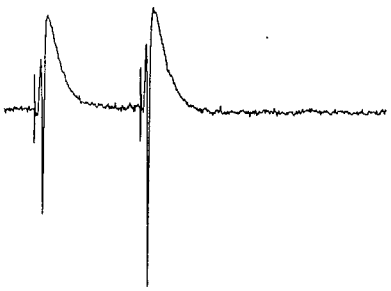


C. Mg^{2+} - FREE + 50 μM OPIPRAMOL, 60 min

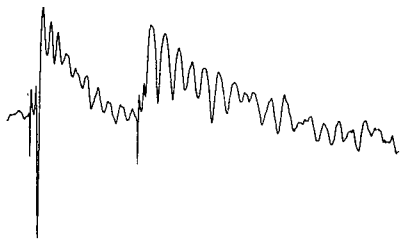
Figure 14. Reversible anticonvulsant effects of opipramol against evoked Mg^{2+}-free epileptiform activity.

A. Electrical stimulation of the SC pathway after a 60 min period of perfusion with Mg^{2+}-containing medium evoked a single population spike following both the first and second stimuli. ***B.*** Epileptiform activity was elicited with paired stimuli after a 60 min exposure to Mg^{2+}-free ACSF. ***C.*** A 60 min period of perfusion with 50 μM opipramol attenuated the epileptiform activity seen in *B*. ***D.*** The Mg^{2+}-free epileptiform response had recovered significantly 60 min after the start of reperfusion with drug-free, Mg^{2+}-free ACSF. ***E.*** After 120 min of drug-free recovery, the epileptiform response to paired stimuli had surpassed the pre-drug epileptiform response seen in B. ***F.*** 180 min following drug washout, the epileptiform response had developed fully. ***G.*** Within 30 min of exposure to Mg^{2+}-containing ACSF the epileptiform responses were completely abolished. All records were obtained from the same hippocampal slice. Scale bars shown in *G* apply to all traces.

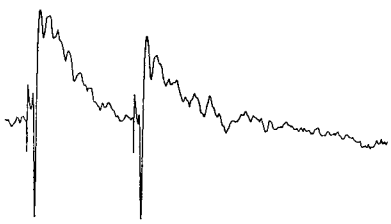
A. Mg^{2+} - CONTAINING CONTROL, 60 min



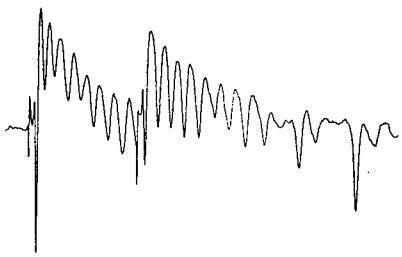
B. Mg^{2+} - FREE, 60 min



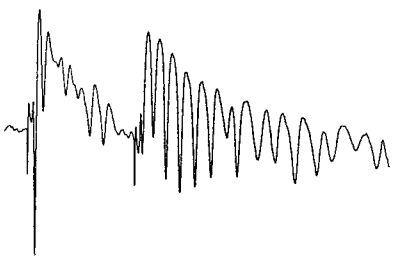
C. Mg^{2+} - FREE + 50 μM OPIPRAMOL, 60 min



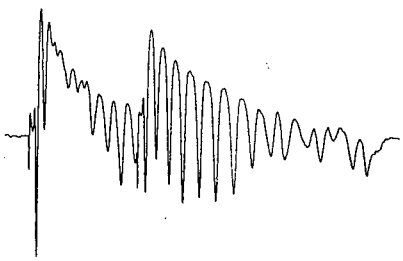
D. Mg^{2+} - FREE RECOVERY, 60 min



E. Mg^{2+} - FREE RECOVERY, 120 min



F. Mg^{2+} - FREE RECOVERY, 180 min



G. Mg^{2+} - CONTAINING RECOVERY, 30 min

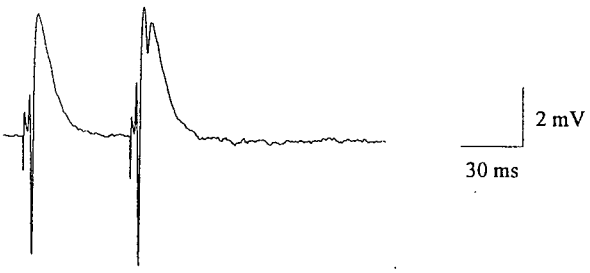


Figure 15. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by opipramol.

IC_{50} value for the inhibition of evoked epileptiform activity by opipramol was 52 μM, with a Hill coefficient of 1.85 and a r^2 value of 0.98. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of opipramol in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

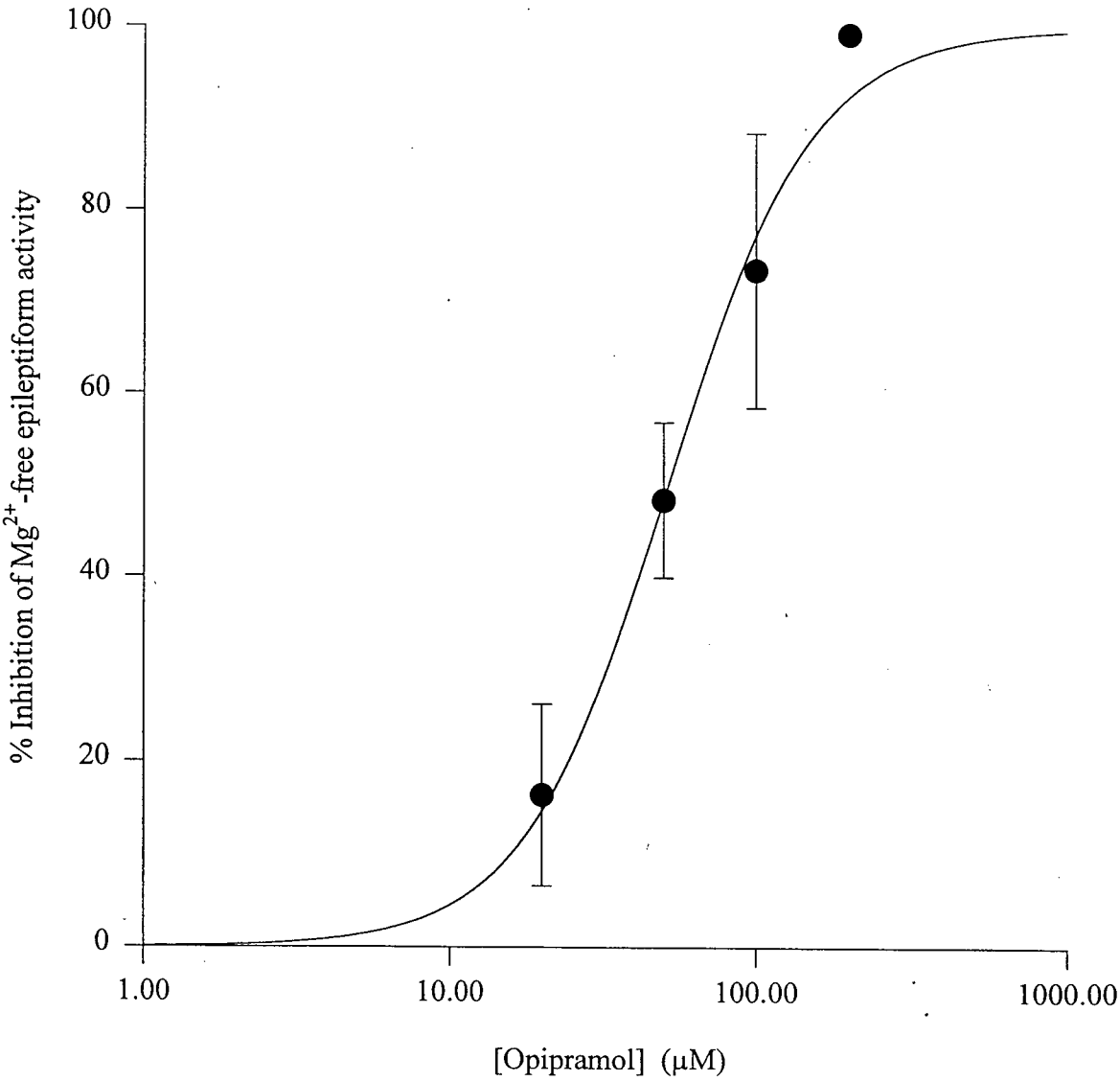
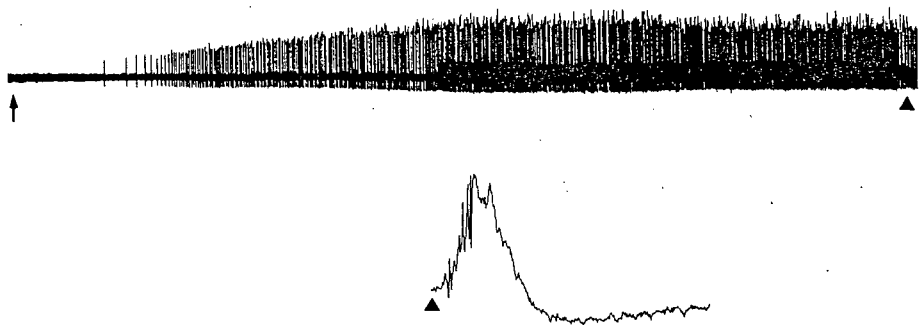

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
1.00
10.00
100.00
1000.00
[Opipramol] (μM)

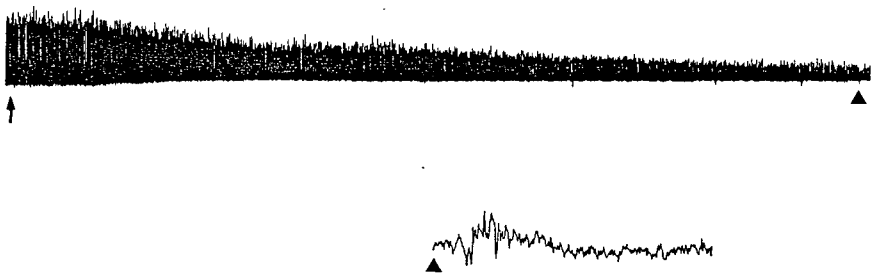
Figure 16. Opipramol reversibly attenuated spontaneous epileptiform activity induced by perfusion with Mg^{2+}-free ACSF.

The lower traces in *A* - *D* represent a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. Arrows represent the initiation of perfusion with the media identified on the figure. ***A.*** Introduction of Mg^{2+}-free ACSF induced spontaneous epileptiform bursts, which attained a stable amplitude and frequency after 60 min. ***B.*** Perfusion with 100 μM opipramol appreciably depressed the amplitude of the spontaneous bursts, as seen in the chart record and the single spontaneous burst shown. ***C.*** Spontaneous burst activity began to recover following a 60 min period of reperfusion with drug-free, Mg^{2+}-free ACSF. ***D.*** At the end of 120 min in drug-free, Mg^{2+}-free ACSF, the amplitude of the bursts had recovered fully, although their frequency was lower than observed prior to the addition of the test compound. All records were obtained from the same hippocampal slice. Scale bars in the upper trace of *D* apply to all chart records. Scale bars for individual spontaneous epileptiform events are also shown in *D*, lower trace.

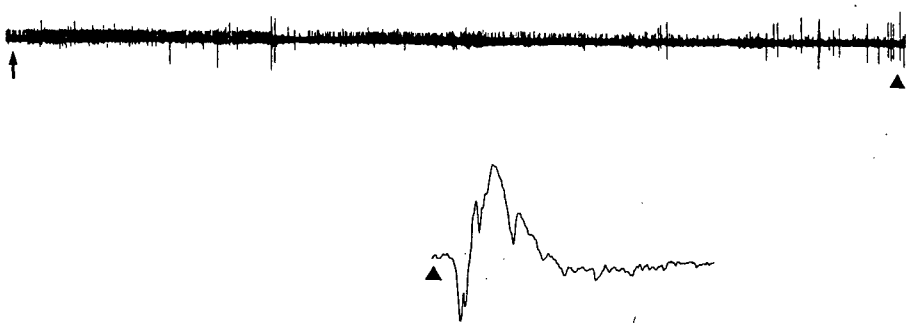
A. Mg^{2+} - FREE, 0 - 60 min



B. Mg^{2+} - FREE + 100 μM OPIPRAMOL, 0 - 60 min



C. Mg^{2+} - FREE RECOVERY, 0 - 60 min



D. Mg^{2+} - FREE RECOVERY, 60 - 120 min

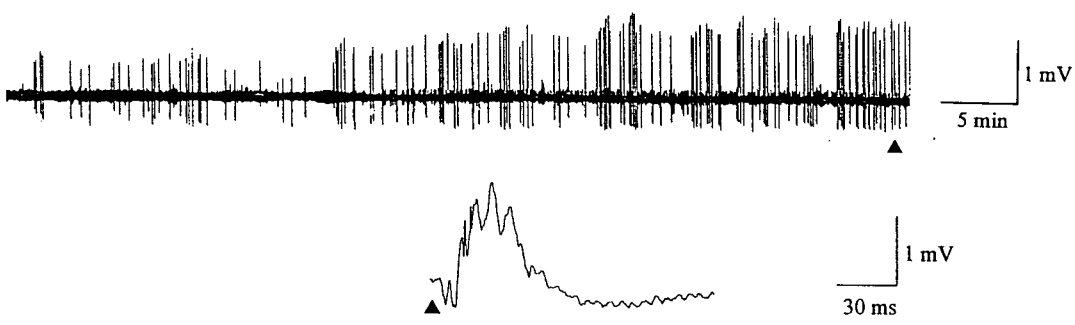
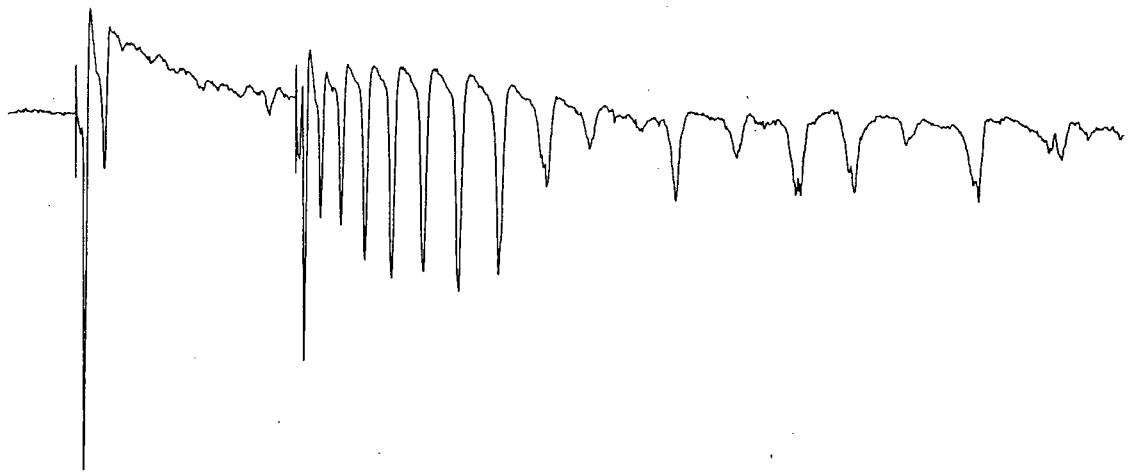


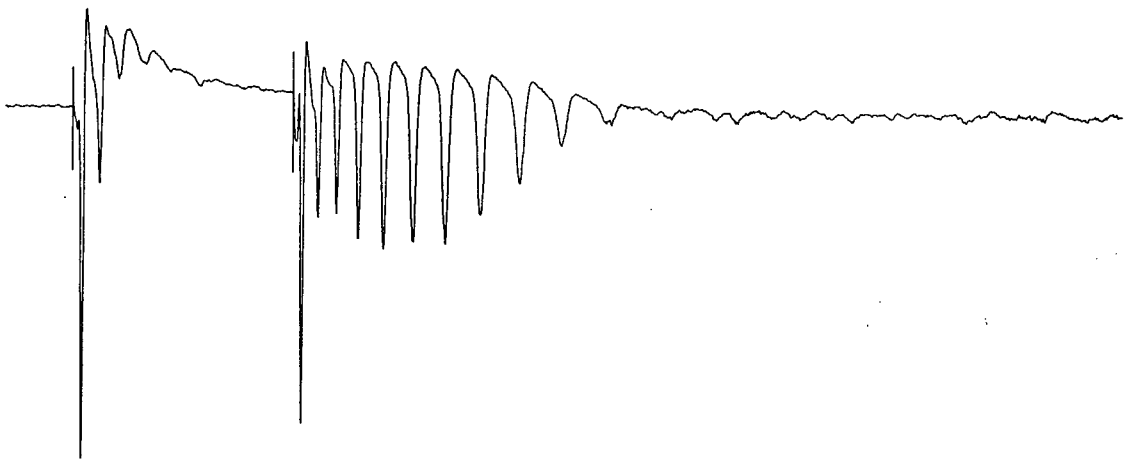
Figure 17. Concentration-dependent inhibition of evoked epileptiform activity by ifenprodil.

A. Perfusion with Mg^{2+}-free ACSF led to the appearance of an epileptiform response when the SC pathway was stimulated with paired stimuli. ***B.*** Addition of 5 μM ifenprodil to the Mg^{2+}-free medium attenuated slightly the evoked epileptiform response after 60 min. ***C.*** Increasing the ifenprodil concentration to 50 μM caused a significant inhibition of evoked epileptiform activity after 60 min. Note the small amplitude of the solitary population spike following the second stimulus (see text). All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all traces.

A. Mg^{2+} - FREE, 60 min



B. Mg^{2+} - FREE + 5 μM IFENPRODIL, 60 min



C. Mg^{2+} - FREE + 50 μM IFENPRODIL, 60 min

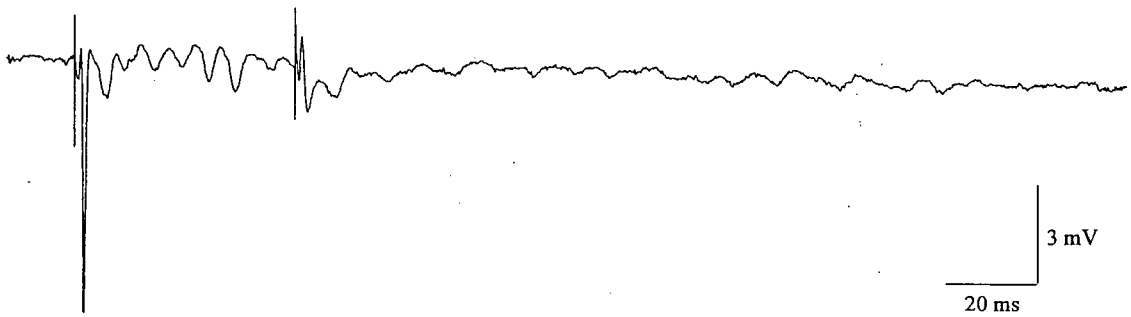


Figure 18. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by ifenprodil.

IC_{50} value for the inhibition of evoked epileptiform activity by ifenprodil was 6 μM, with a Hill coefficient of 4.81 and a r^2 value of 0.97. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of ifenprodil in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

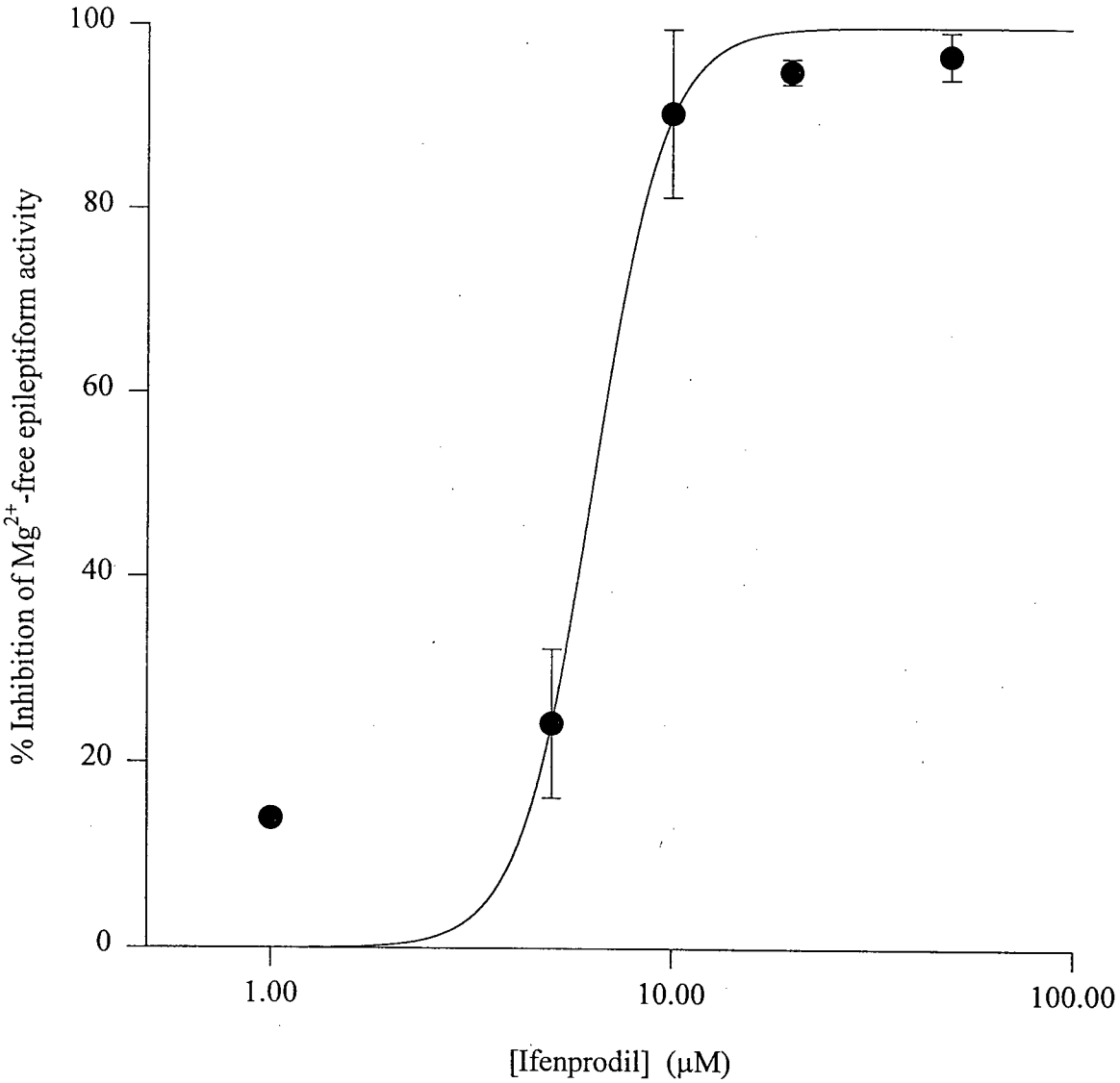
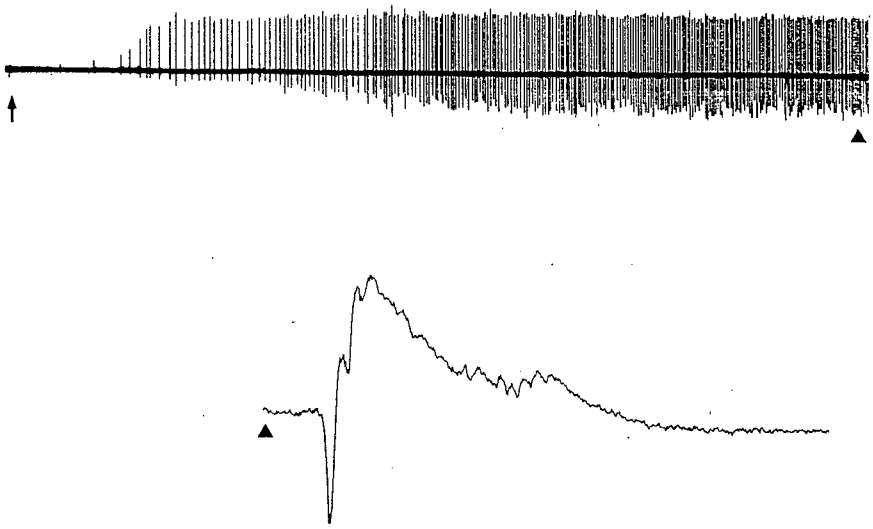

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
1.00
10.00
100.00
[Ifenprodil] (μM)

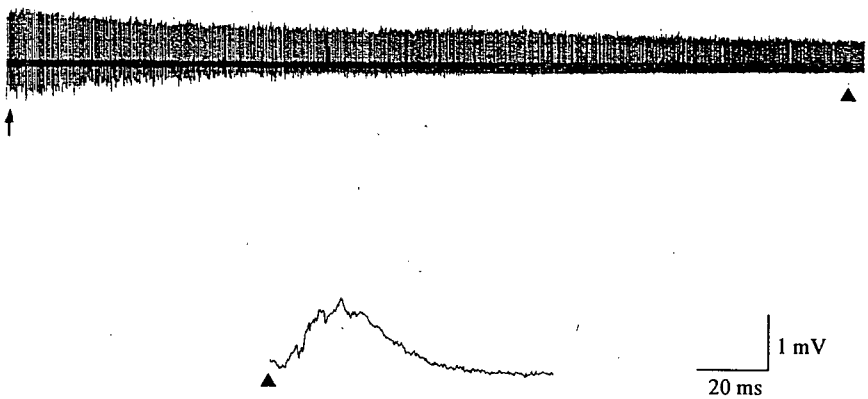
Figure 19. Spontaneous epileptiform activity was attenuated in a concentration-dependent manner by ifenprodil.

The lower traces in *A* and *B* represent a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. The absence of a representative single spontaneous burst in *C* indicates that spontaneous activity was abolished, as can be seen in the accompanying chart record. Arrows below each chart record represent the initiation of perfusion with the media identified on the figure. ***A.*** Spontaneous epileptiform bursts developed with the introduction of Mg^{2+}-free ACSF and reached a consistent amplitude and frequency after 60 min. ***B.*** Following a 60 min period of perfusion with 5 μM ifenprodil the spontaneous bursts were decreased in amplitude, as seen in the chart record and the single spontaneous burst shown. ***C.*** Exposure to 20 μM ifenprodil completely abolished the spontaneous epileptiform activity after a 37 min period of perfusion. All records were obtained from the same hippocampal slice. Scale bars in *C* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *B*.

A. Mg^{2+} - FREE, 0 - 60 min



B. Mg^{2+} - FREE + 5 μM IFENPRODIL, 0 - 60 min

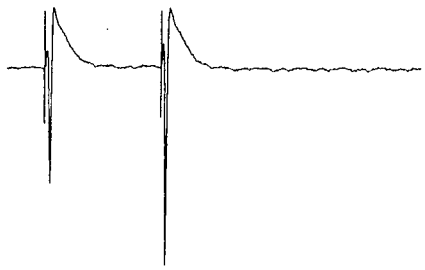


C. Mg^{2+} - FREE + 20 μM IFENPRODIL, 0 - 60 min

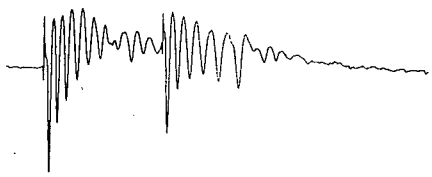
Figure 20. Reversible anticonvulsant effect of dextromethorphan (DXM) against evoked Mg^{2+}-free epileptiform activity.

A. Electrical stimulation of the SC pathway after a 60 min period of perfusion with Mg^{2+}-containing medium evoked a single population spike following both the first and second stimuli. ***B.*** Following a 60 min period of perfusion with Mg^{2+}-free medium, paired stimuli evoked multiple population spikes following both the first and second stimuli. ***C.*** After the slice was exposed to 50 μM DXM, the epileptiform response seen in *B* was attenuated markedly. ***D.*** No recovery of the epileptiform response was observed after 60 min of reperfusion with drug-free, Mg^{2+}-free medium. ***E.*** 120 min after the return to Mg^{2+}-free medium, no recovery of the epileptiform response was seen. ***F.*** Evoked epileptiform activity began to recover at 240 min following drug washout. ***G.*** After 360 min in Mg^{2+}-free medium, evoked epileptiform activity had recovered to the levels observed prior to drug exposure. ***H.*** Return to normal (Mg^{2+}-containing) medium for 40 min resulted in almost complete inhibition of the epileptiform response. All records were obtained from the same hippocampal slice. Scale bars shown in *H* apply to all traces.

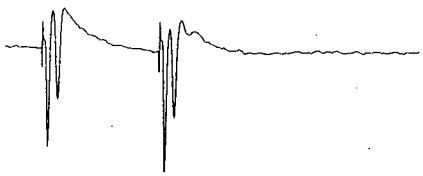
A. Mg^{2+} - CONTAINING CONTROL, 60 min



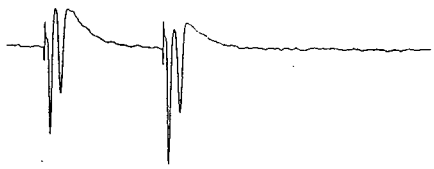
B. Mg^{2+} - FREE, 60 min



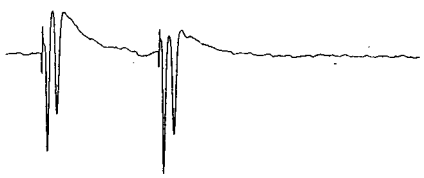
C. Mg^{2+} - FREE + 50 μM DXM, 60 min



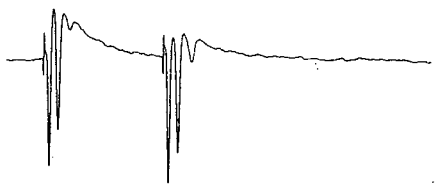
D. Mg^{2+} - FREE RECOVERY, 60 min



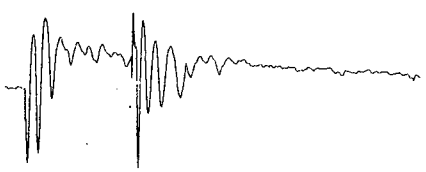
E. Mg^{2+} - FREE RECOVERY, 120 min



F. Mg^{2+} - FREE RECOVERY, 240 min



G. Mg^{2+} - FREE RECOVERY, 360 min



H. Mg^{2+} - CONTAINING RECOVERY, 40 min

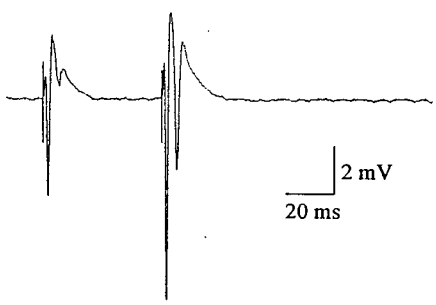


Figure 21. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by DXM.

IC_{50} value for the inhibition of evoked epileptiform activity by DXM was 10 μM with a Hill coefficient of 0.89 and a r^2 value of 0.78. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of DXM in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

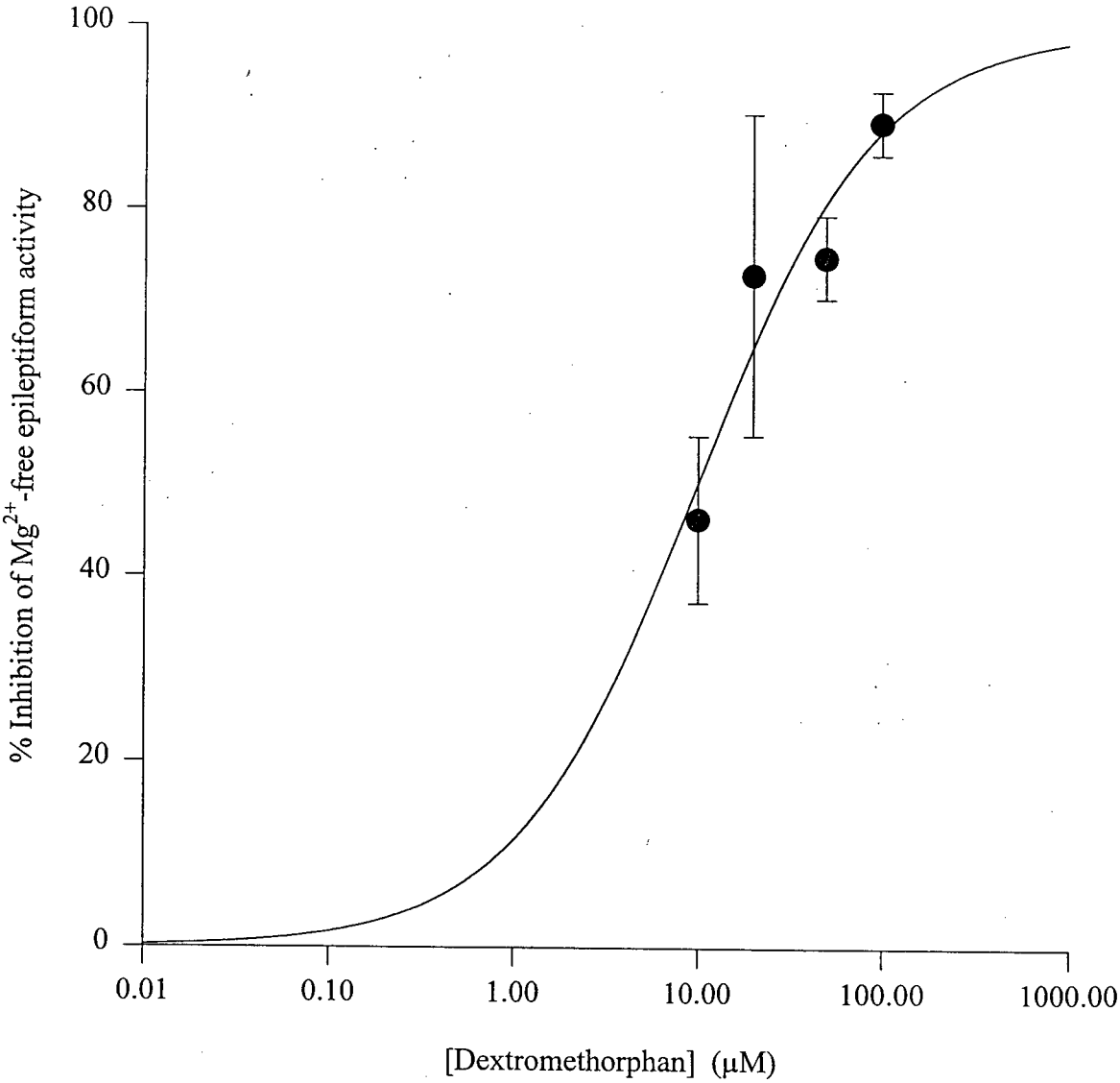
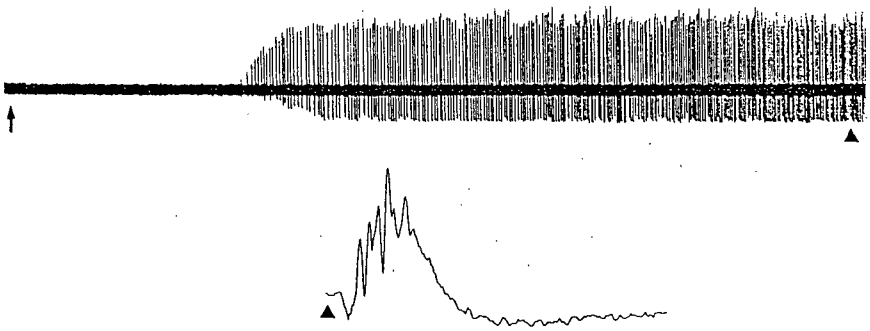

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
0.01
0.10
1.00
10.00
100.00
1000.00
[Dextromethorphan] (μM)

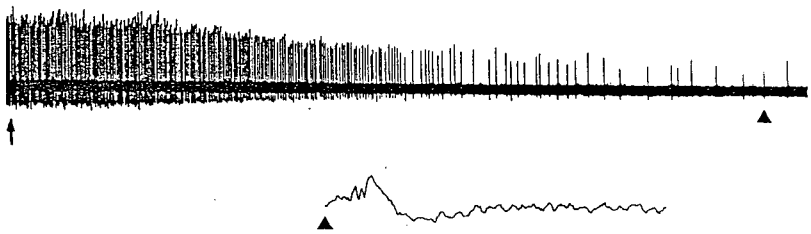
Figure 22. DXM reversibly inhibited spontaneous epileptiform activity induced by perfusion with Mg^{2+}-free ACSF.

The lower trace in each figure represents a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. Arrows beneath the chart recorder traces indicate the initiation of perfusion with the media identified on the figure. ***A.*** Following the introduction of Mg^{2+}-free ACSF, spontaneous epileptiform bursts developed, reaching a consistent amplitude and frequency after 60 min. ***B.*** Perfusion with 50 μM DXM decreased both the amplitude and frequency of the spontaneous epileptiform bursts after 60 min, as shown in the chart record and single spontaneous burst. ***C.*** Mg^{2+}-free spontaneous bursts showed little recovery between 60 - 120 min following drug washout. ***D.*** The frequency and amplitude of the spontaneous bursts were both increased between 180 - 240 min of reperfusion with drug-free, Mg^{2+}-free medium. ***E.*** The amplitude of the spontaneous epileptiform bursts attained pre-drug values at 300 min following removal of the drug. All records were obtained from the same hippocampal slice. Scale bars in the upper trace of *E* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *E*, lower trace.

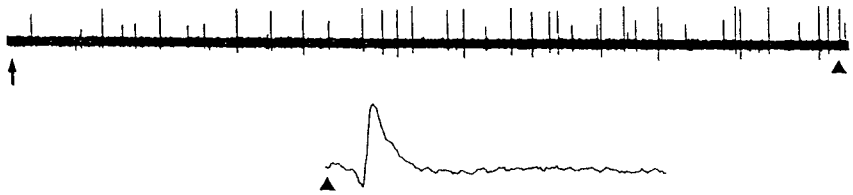
A. Mg^{2+} - FREE, 0 - 60 min



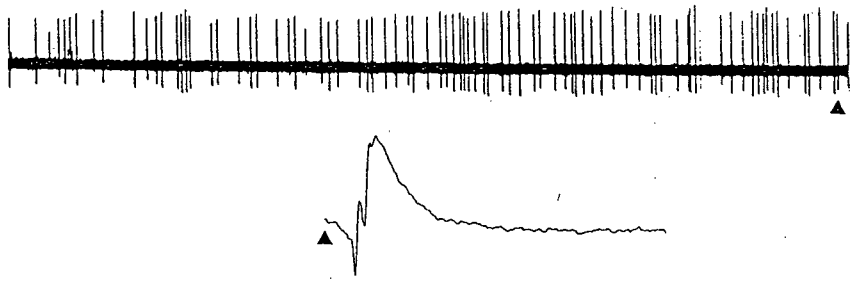
B. Mg^{2+} - FREE + 50 μM DXM, 0 - 60 min



C. Mg^{2+} - FREE RECOVERY, 60 - 120 min



D. Mg^{2+} - FREE RECOVERY, 180 - 240 min



E. Mg^{2+} - FREE RECOVERY, 240 -300 min

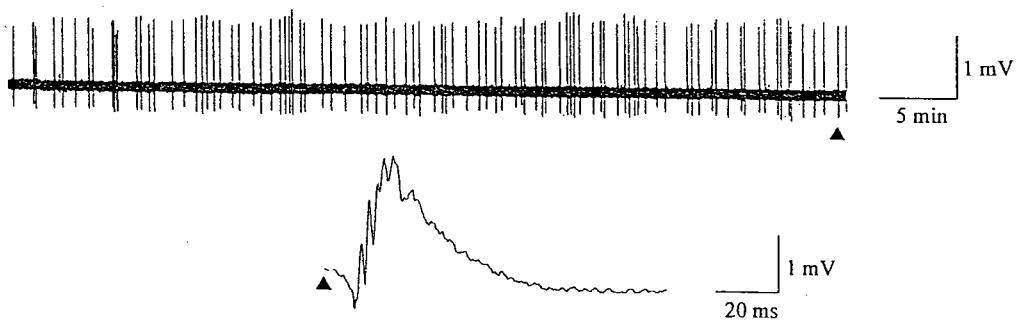
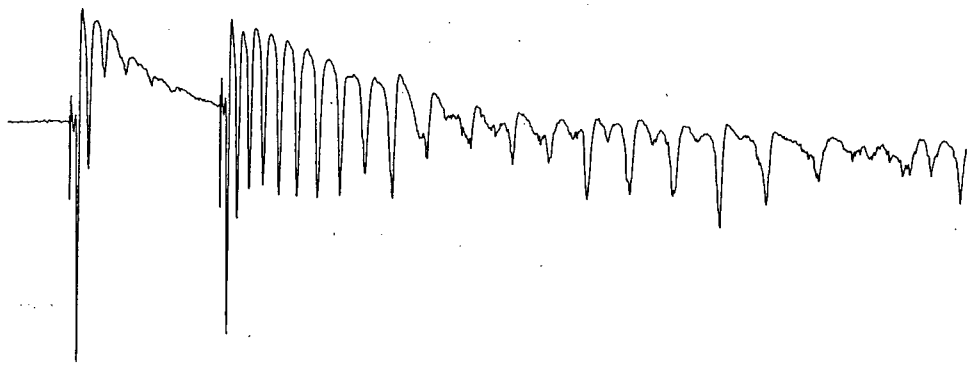


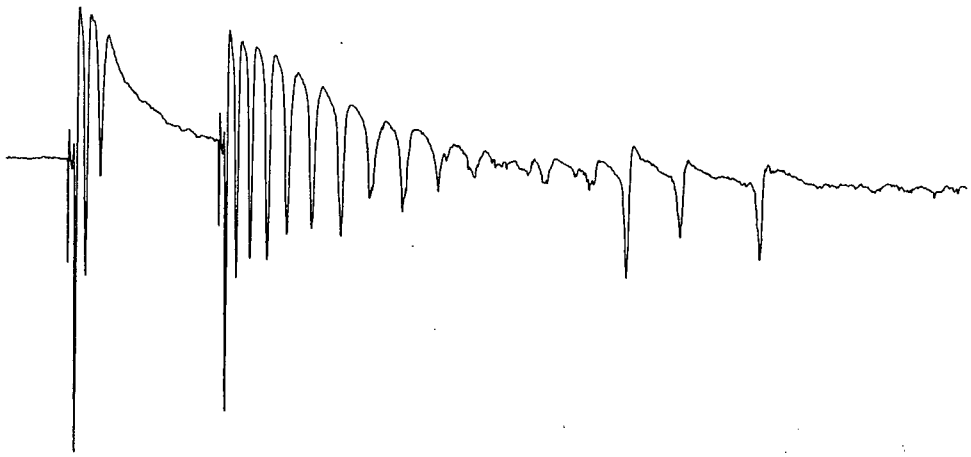
Figure 23. 1,3-di(2-tolyl)guanidine (DTG) inhibited evoked epileptiform responses in a concentration-dependent manner.

A. Epileptiform activity was evoked with paired stimuli following a 60 min exposure to Mg^{2+}-free ACSF. ***B.*** A 60 min exposure to 10 μM DTG decreased slightly the number of population spikes following both the first and second stimuli. ***C.*** Following the period of perfusion with 10 μM DTG, the concentration of DTG was increased to 100 μM. After 60 min of perfusion the epileptiform response was reduced markedly. All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all traces.

A. Mg^{2+} - FREE, 60 min



B. Mg^{2+} - FREE + 10 μM DTG, 60 min



C. Mg^{2+} - FREE + 100 μM DTG, 60 min

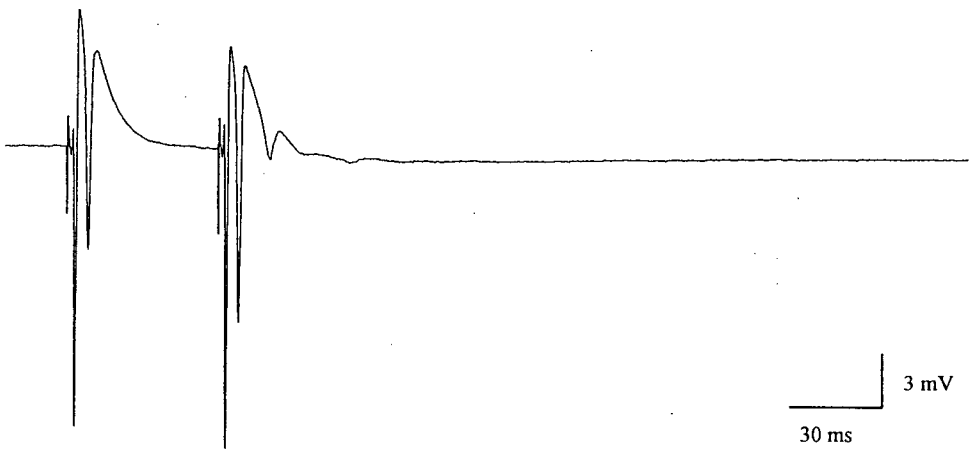
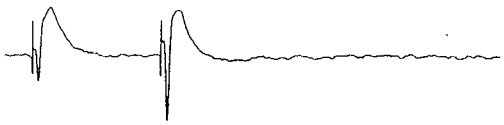


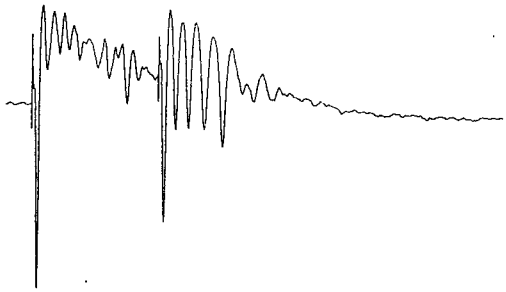
Figure 24. Reversible anticonvulsant effect of DTG against evoked Mg^{2+}-free epileptiform activity.

A. Evoked response to paired stimuli following a 60 min period of perfusion with normal (Mg^{2+}-containing) ACSF. Note the single population spike following either the first or second stimulus. ***B.*** Perfusion with Mg^{2+}-free ACSF for 60 min resulted in epileptiform activity. ***C.*** A 60 min exposure to 50 μM DTG almost abolished the epileptiform response seen in *B*. ***D.*** 60 min following return to drug-free, Mg^{2+}-free ACSF, the epileptiform activity began to recover. ***E.*** Mg^{2+}-free evoked epileptiform activity continued to recover at 120 min following drug treatment. ***F.*** At 180 min after the return to drug-free, Mg^{2+}-free medium, the evoked epileptiform response had almost recovered back to pre-drug levels seen in *B*. ***G.*** Return to Mg^{2+}-containing ACSF abolished the epileptiform activity after 60 min. All records were obtained from the same hippocampal slice. Scale bars shown in *G* apply to all traces.

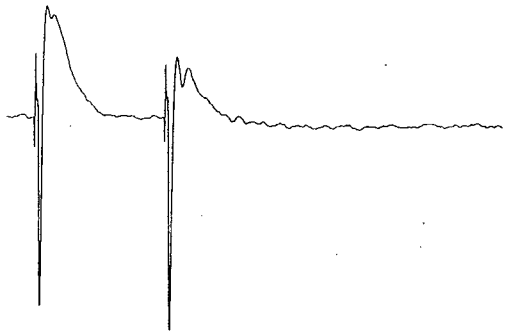
A. Mg^{2+} - CONTAINING CONTROL, 60 min



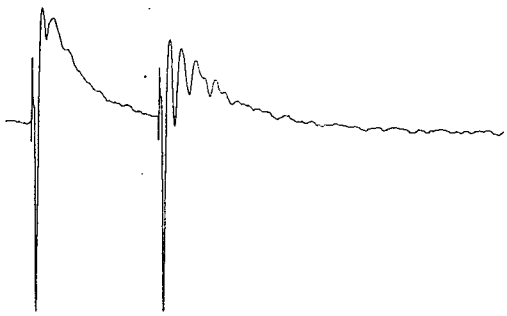
B. Mg^{2+} - FREE, 60 min



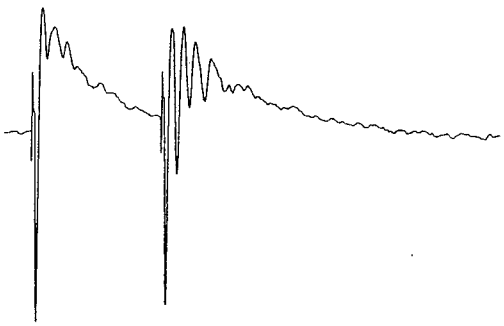
C. Mg^{2+} - FREE + 50 μM DTG, 60 min



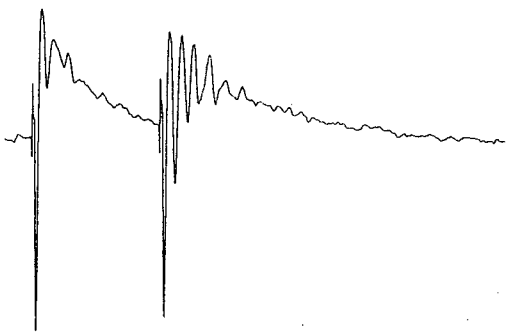
D. Mg^{2+} - FREE RECOVERY, 60 min



E. Mg^{2+} - FREE RECOVERY, 120 min



F. Mg^{2+} - FREE RECOVERY, 180 min



G. Mg^{2+} - CONTAINING RECOVERY, 60 min

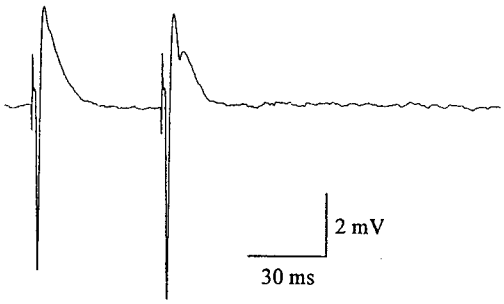


Figure 25. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by DTG.

IC_{50} value for the inhibition of evoked epileptiform activity by DTG was 15 μM, with a Hill coefficient of 2.93 and a r^2 value of 0.99. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of DTG in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

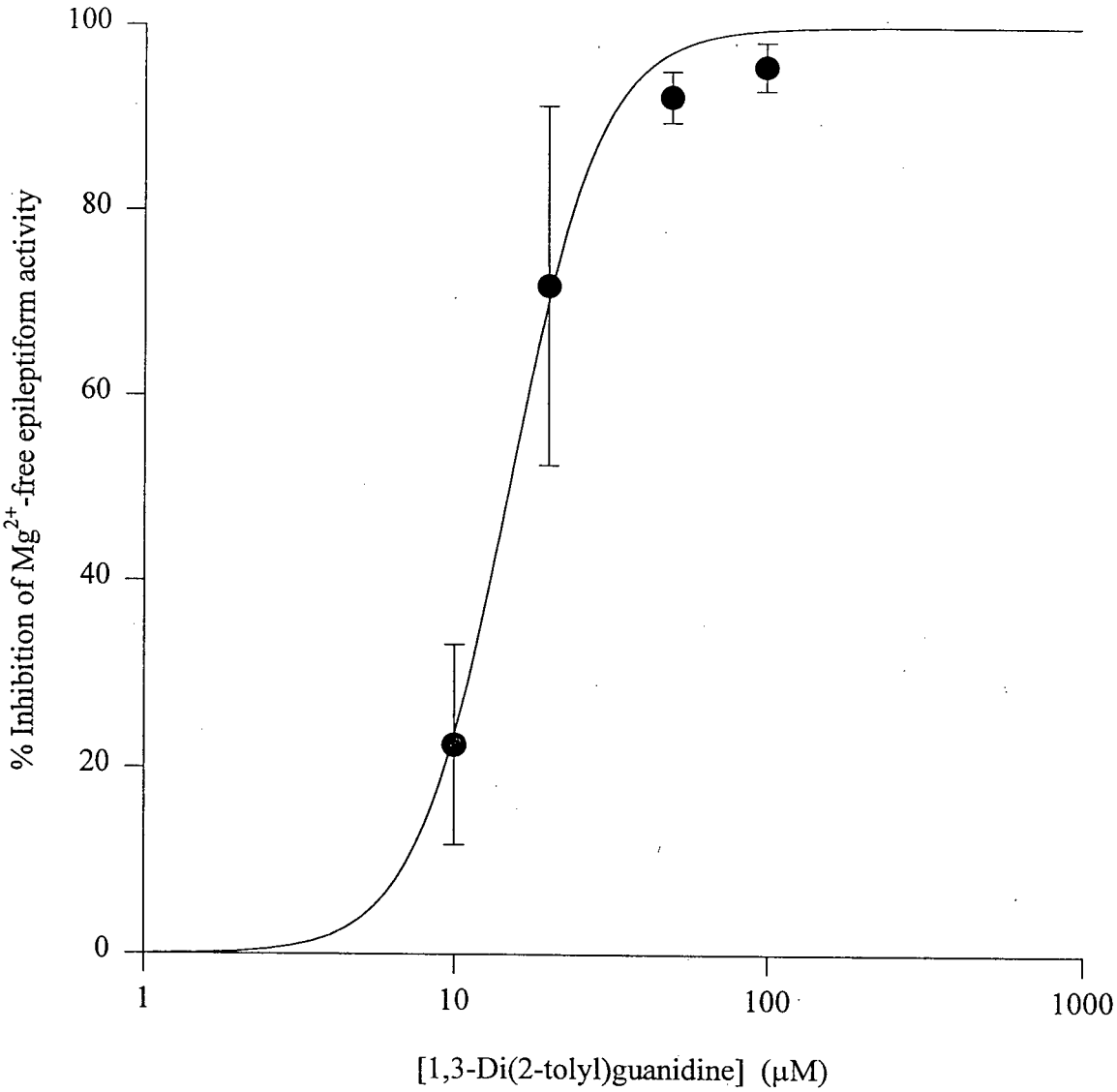

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
1
10
100
1000
[1,3-Di(2-tolyl)guanidine] (μM)

Figure 26. Superimposition of extracellularly recorded responses to paired stimuli in Mg^{2+}-free (solid trace) and Mg^{2+}-free + DTG-containing (dotted trace) ACSF.

Low concentrations of DTG, although anticonvulsant in their overall effect, occasionally caused an increase in the amplitude of the first (and sometimes subsequent) population spikes. In the dotted trace, note the increased amplitude of the first population spike following both the first and second stimuli, accompanied by a decrease in the number and amplitude of subsequent population spikes. Each record was obtained from the same hippocampal slice. Scale bars shown apply to both traces.

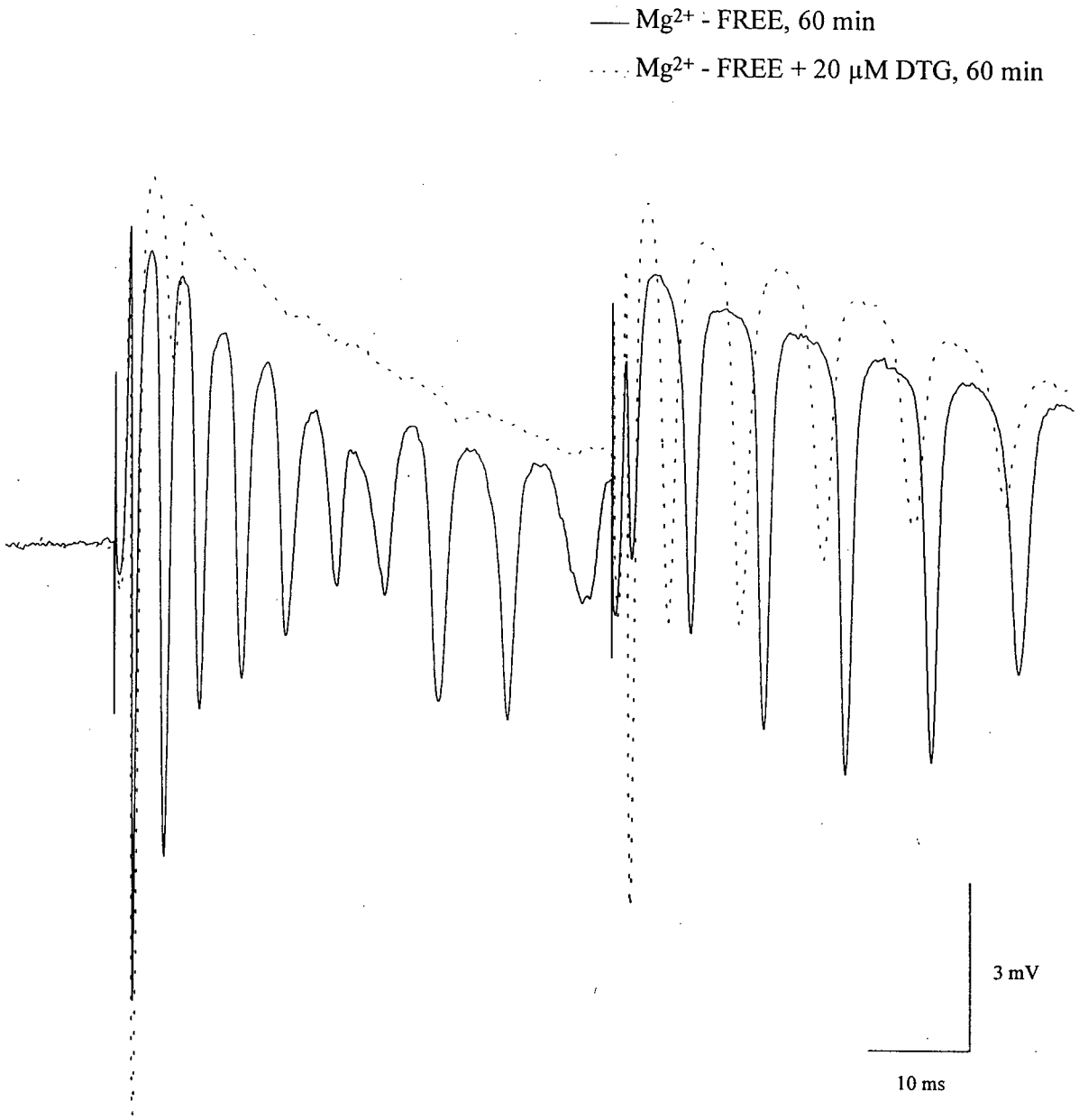
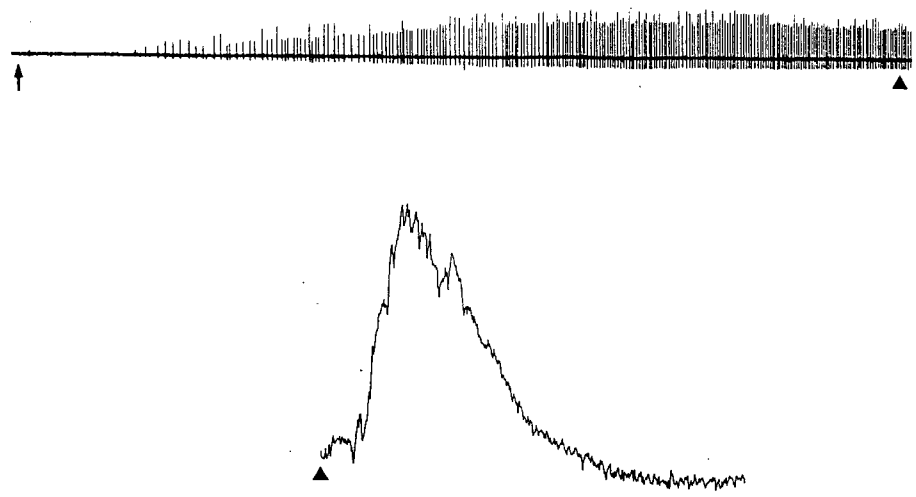

— Mg^{2+} - FREE, 60 min
. . . . Mg^{2+} - FREE + 20 μM DTG, 60 min
3 mV
10 ms

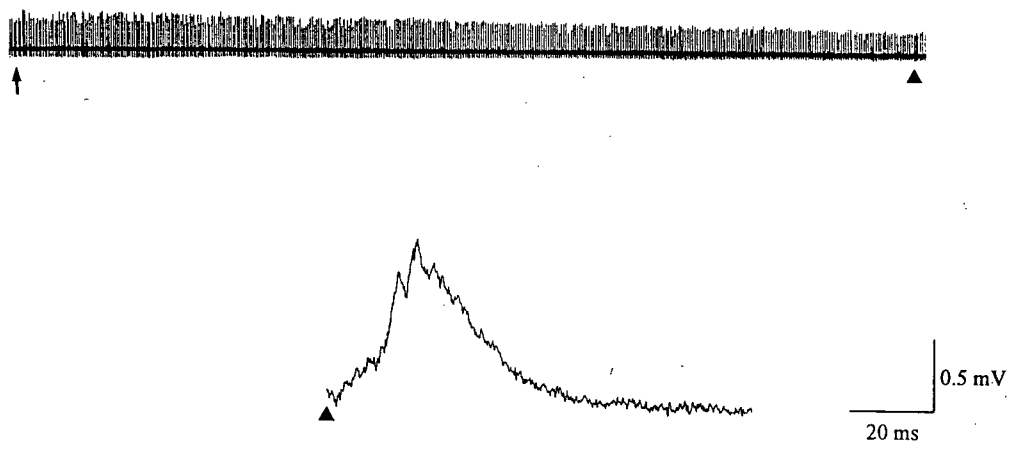
Figure 27. Spontaneous epileptiform bursting was reduced in a concentration-dependent manner by DTG.

The lower traces in *A* and *B* represent a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. The absence of a representative single spontaneous burst in *C* indicates that spontaneous activity was abolished, as can be seen in the accompanying chart record. Arrows beneath the chart records represent the initiation of perfusion with the media identified on the figure. ***A.*** Spontaneous epileptiform bursts reached a consistent amplitude and frequency after a 60 min period of perfusion with Mg^{2+}-free ACSF. ***B.*** Perfusion with 10 μM DTG gradually decreased the amplitude and frequency of spontaneous bursts over a 60 min period. ***C.*** After a 55 min exposure to 100 μM DTG, spontaneous epileptiform activity was completely abolished. All records were obtained from the same hippocampal slice. Scale bars in *C* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *B*.

A. Mg^{2+} - FREE, 0 - 60 min



B. Mg^{2+} - FREE + 10 μM DTG, 0 - 60 min



C. Mg^{2+} - FREE + 100 μM DTG, 0 - 60 min

Figure 28. DTG reversibly inhibited spontaneous epileptiform activity induced by perfusion with Mg^{2+}-free ACSF.

The lower trace in each section of the figure represents a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. Arrows beneath the chart records represent the initiation of perfusion with the media identified on the figure. ***A.*** Introduction of Mg^{2+}-free ACSF initiated spontaneous epileptiform bursts, which attained a consistent amplitude and frequency after 60 min. ***B.*** Perfusion with 50 μM DTG decreased both the amplitude and frequency of the epileptiform bursts. ***C.*** 60 min after drug washout, Mg^{2+}-free spontaneous bursts had increased in amplitude and frequency. ***D.*** After 120 min in Mg^{2+}-free medium, spontaneous epileptiform bursts continued to increase in frequency, while their amplitude remained constant. ***E.*** The amplitude and frequency of the epileptiform bursts reached a stable level of recovery 180 min after the start of reperfusion with Mg^{2+}-free, drug-free perfusate. All records were obtained from the same hippocampal slice. Scale bars in the upper trace of *E* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *E*, lower trace.

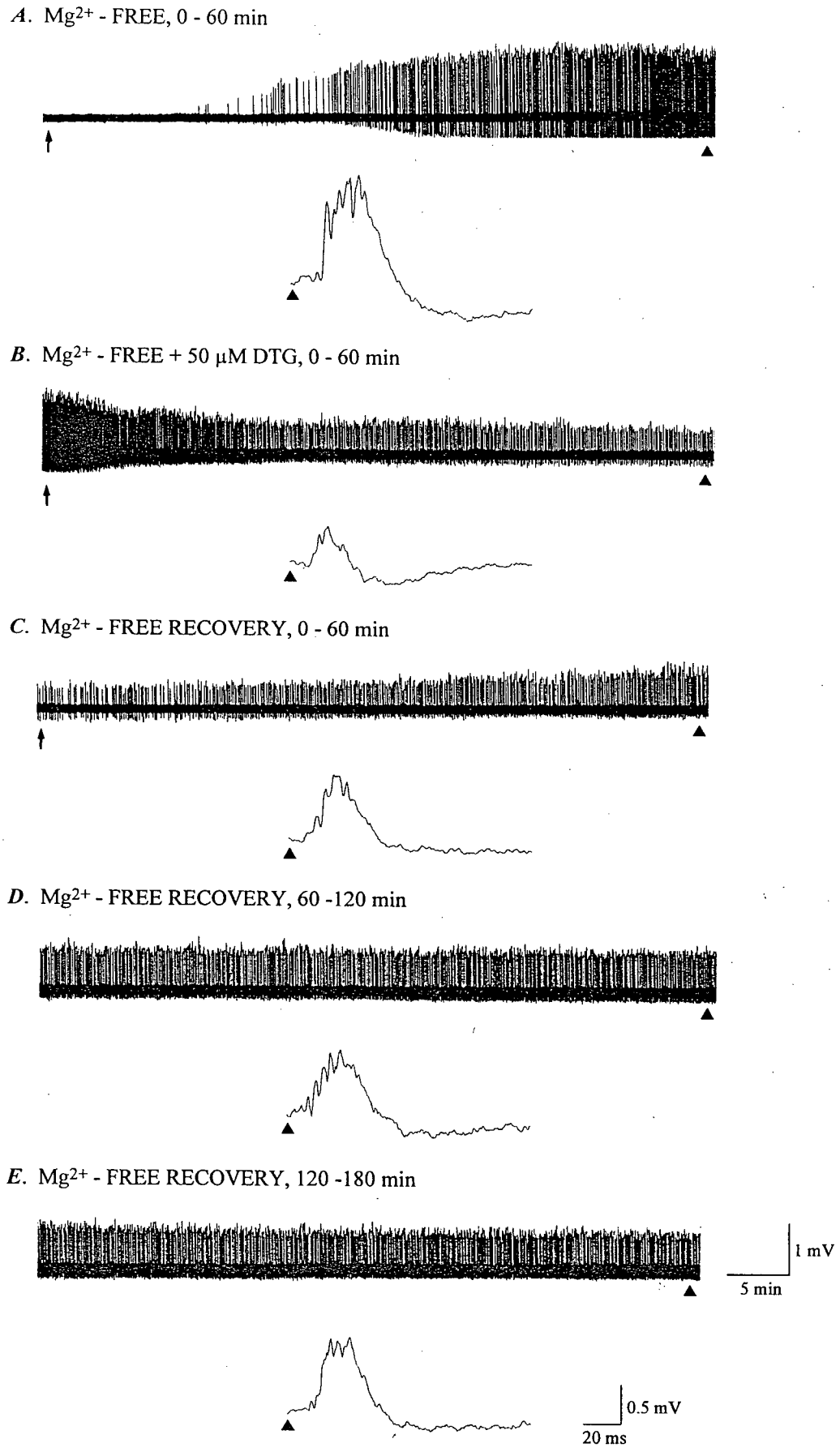
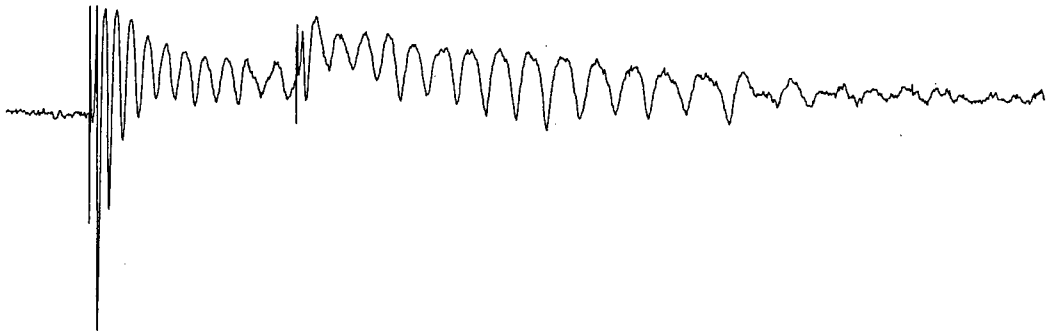

A. Mg2+ - FREE, 0 - 60 min
B. Mg2+ - FREE + 50 µM DTG, 0 - 60 min
C. Mg2+ - FREE RECOVERY, 0 - 60 min
D. Mg2+ - FREE RECOVERY, 60 -120 min
E. Mg2+ - FREE RECOVERY, 120 -180 min
1 mV
5 min
0.5 mV
20 ms

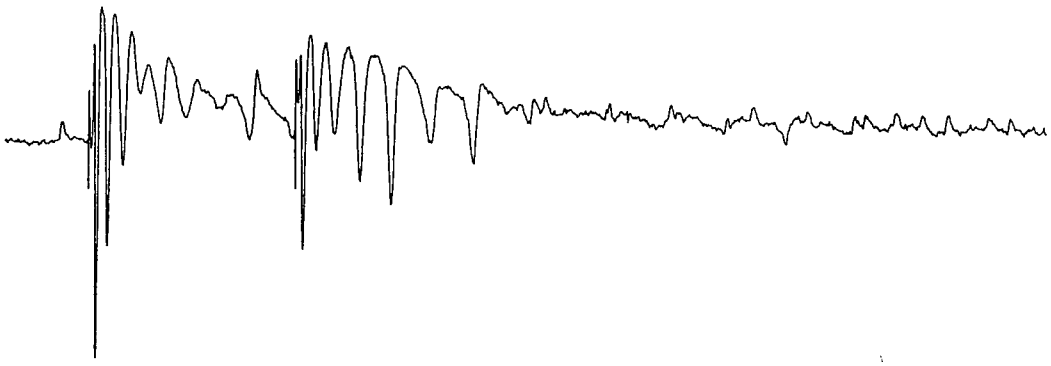
Figure 29. Concentration-dependent inhibition of evoked epileptiform activity by dextrorphan (DX).

A. Following a 60 min period of perfusion with Mg^{2+}-free ACSF, paired stimulation of the SC pathway evoked epileptiform activity. ***B.*** Exposure to 2 μM DX attenuated slightly the number of population spikes, notably those evoked by the second stimulus. ***C.*** 60 min after the start of perfusion with 20 μM DX, the number of population spikes following paired stimuli was significantly decreased. All records were obtained from the same hippocampal slice. Scale bars shown in *C* apply to all traces.

A. Mg^{2+} - FREE, 60 min



B. Mg^{2+} - FREE + 2 μM DEXTRORPHAN, 60 min



C. Mg^{2+} - FREE + 20 μM DEXTRORPHAN, 60 min

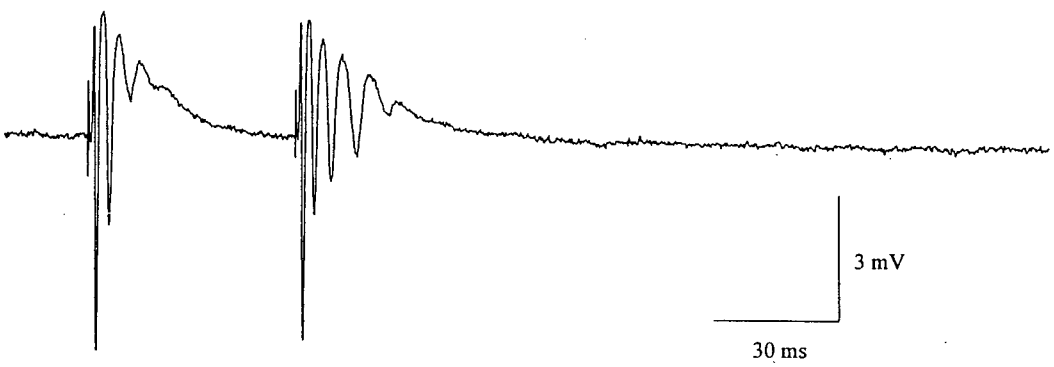


Figure 30. Log concentration-inhibition plot for inhibition of Mg^{2+}-free evoked epileptiform activity by DX.

IC_{50} value for the inhibition of evoked epileptiform activity by DX was 2 μM with a Hill coefficient of 0.93 and a r^2 value of 0.95. Data points indicate mean ± s.e.m for 2 - 5 experiments. Abscissa scale: concentration of DX in μM. Ordinate scale: % inhibition of evoked epileptiform activity.

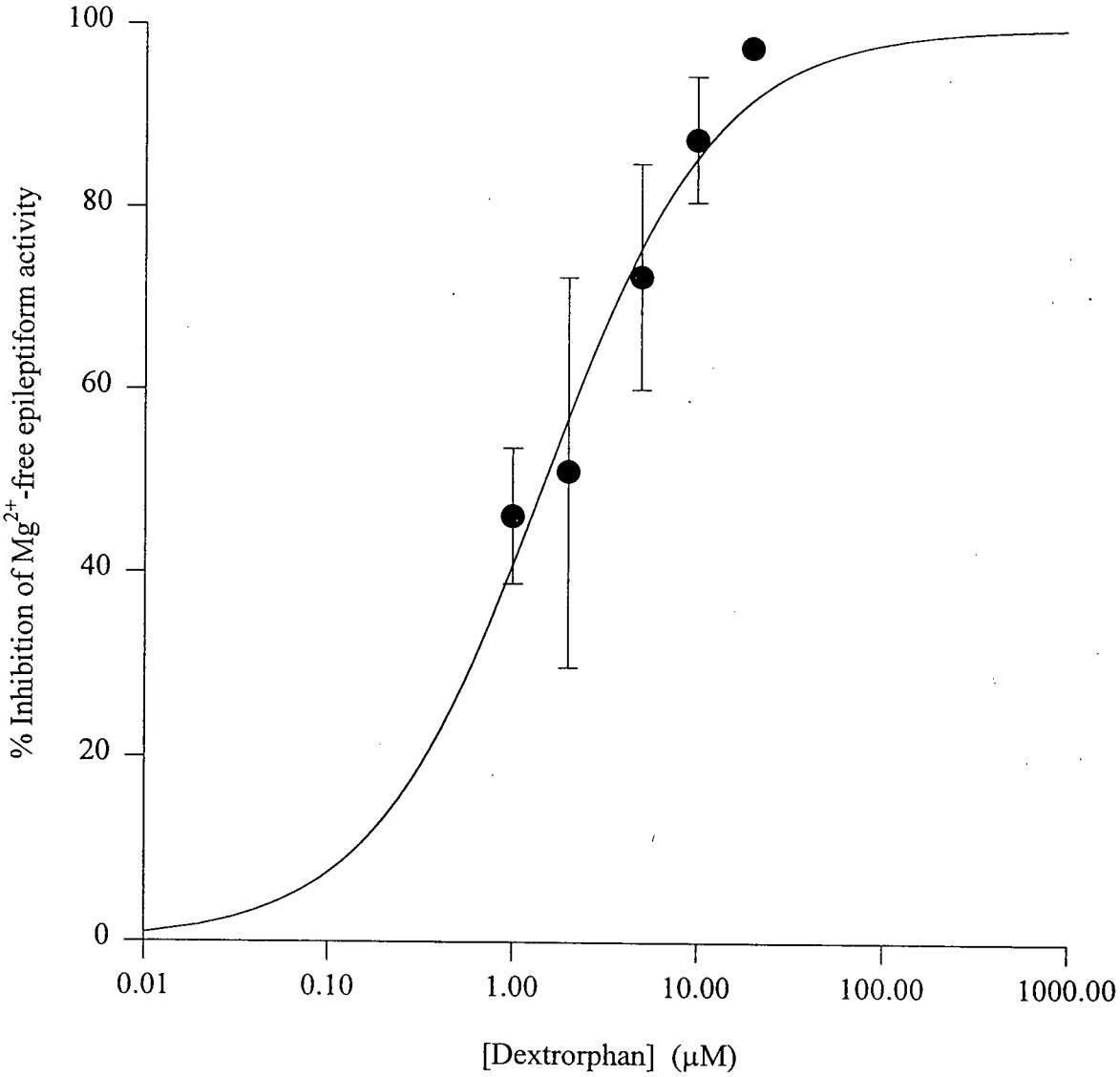

100
80
60
40
20
0
% Inhibition of Mg^{2+}-free epileptiform activity
0.01
0.10
1.00
10.00
100.00
1000.00
[Dextrorphan] (μM)

Figure 31. Superimposition of extracellularly recorded responses to paired stimuli in Mg^{2+}-free (solid trace) and Mg^{2+}-free + DX-containing (dotted trace) ACSF.

Low concentrations of DX, although anticonvulsant in their overall effect, occasionally caused an increase in the amplitude of the first (and sometimes subsequent) population spikes. In the dotted trace, note the increased amplitude of the first two to three population spikes following both the first and second stimuli, accompanied by a decreased number and amplitude of subsequent population spikes. Each record was obtained from the same hippocampal slice. Scale bars shown apply to both traces.

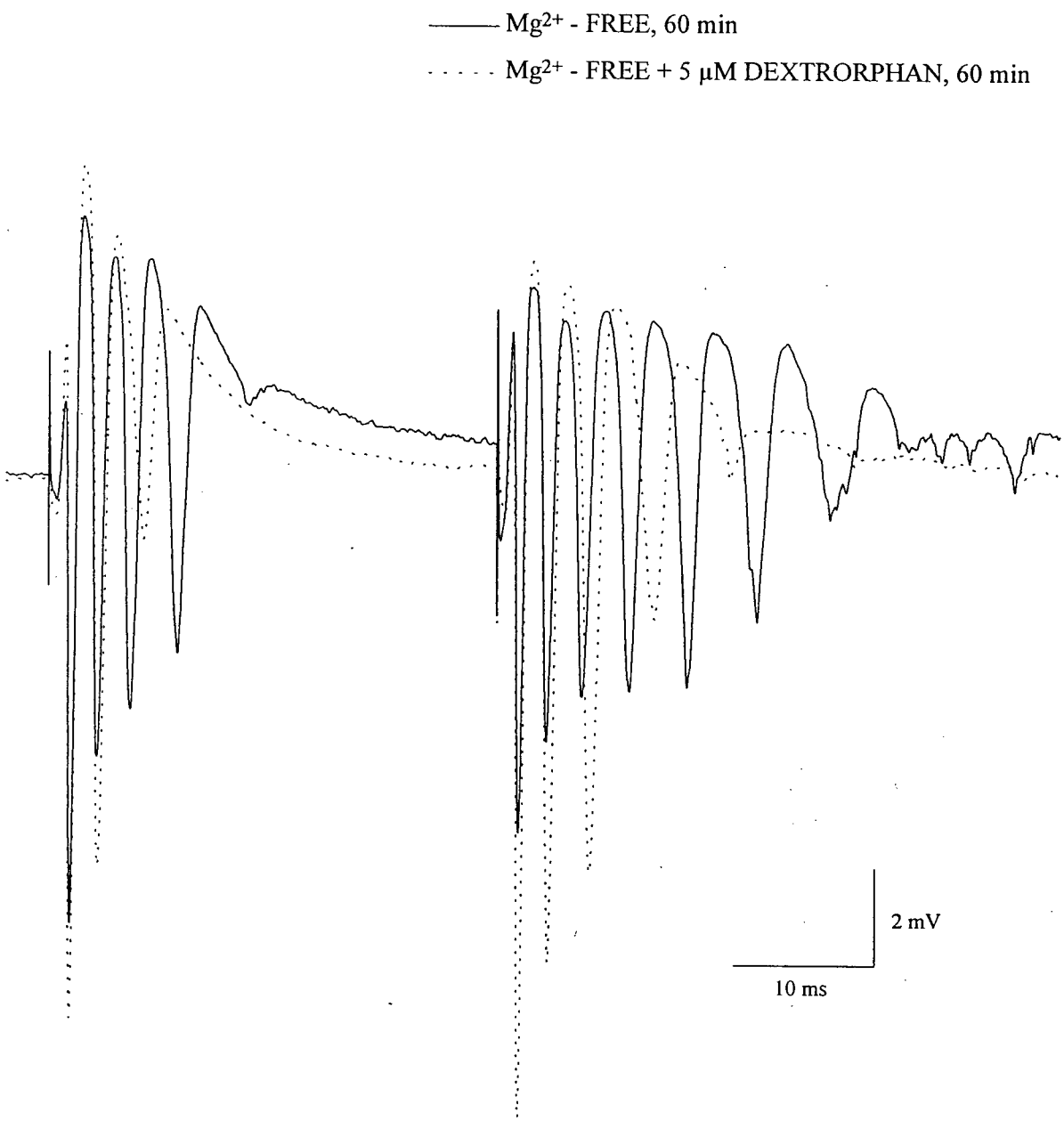
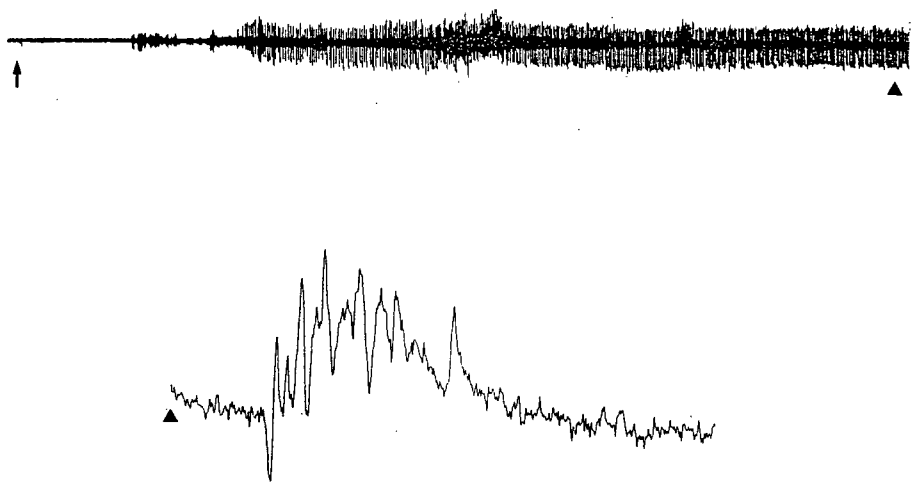

Mg^{2+} - FREE, 60 min
Mg^{2+} - FREE + 5 μM DEXTRORPHAN, 60 min
2 mV
10 ms

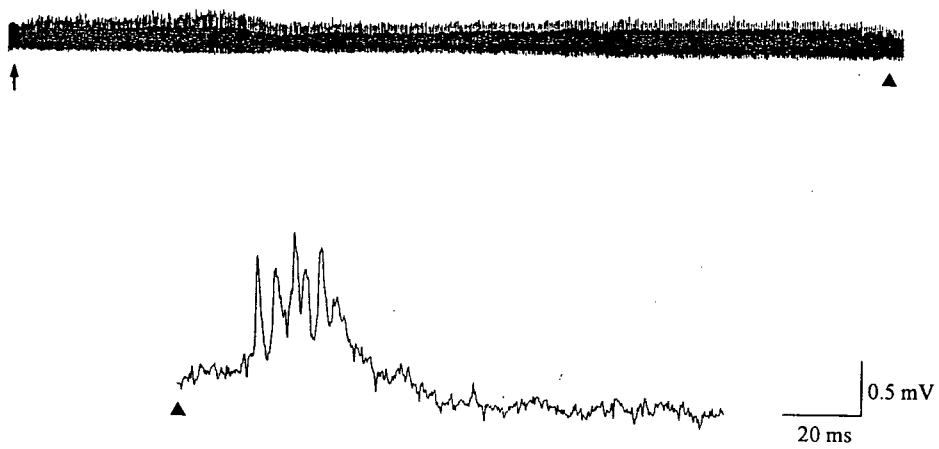
Figure 32. Concentration-dependent inhibition of Mg^{2+}-free spontaneous epileptiform activity by DX.

The lower traces in *A* and *B* represent a single spontaneous burst captured at the corresponding symbol (▲) in the chart recorder trace above. The absence of a representative single spontaneous burst in C indicates that spontaneous activity was completely abolished, as can be seen in the accompanying chart record. Arrows beneath the chart records represent the initiation of perfusion with the media identified on the figure. ***A.*** Spontaneous epileptiform bursts developed following the introduction of Mg^{2+}-free ACSF at the arrow. After 60 min, the bursts attained a consistent amplitude and frequency. ***B.*** A 60 min period of perfusion with 2 μM DX induced a slight decrease in the amplitude and frequency of the spontaneous bursts. ***C.*** Exposure to 20 μM DX completely abolished spontaneous epileptiform activity after a 55 min period of perfusion. All records were obtained from the same hippocampal slice. Scale bars in *C* apply to all chart records. Scale bars for individual spontaneous epileptiform events are shown in *B*.

A. Mg^{2+} - FREE, 0 - 60 min



B. Mg^{2+} - FREE + 2 μM DEXTRORPHAN, 0 - 60 min

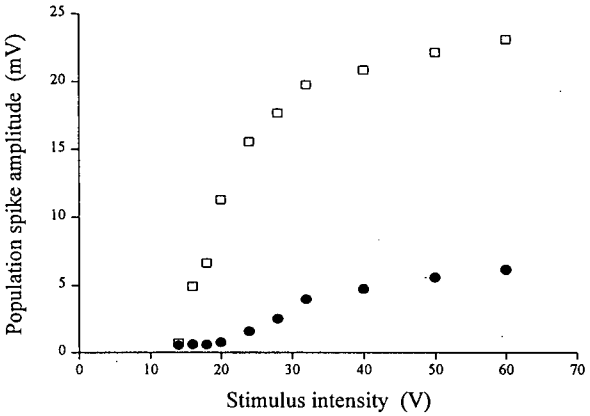


C. Mg^{2+} - FREE + 20 μM DEXTRORPHAN, 0 - 60 min

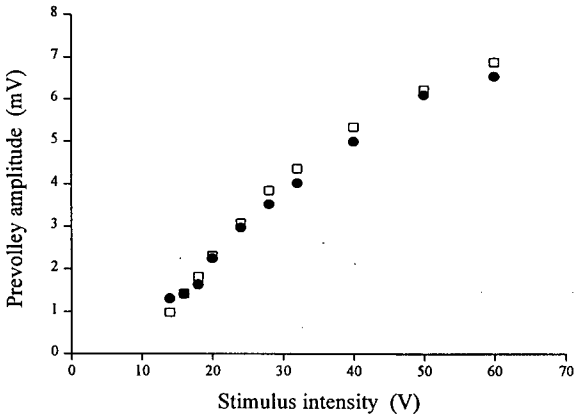
Figure 33. Results of a typical experiment demonstrating adenosine's effects on input/output (I/O) functions along the CA3-CA1 pathway in the hippocampal slice.

A. In the presence of 300 μM adenosine (●) an inhibitory effect on the overall I/O function of the pathway was observed when compared to control (□) responses. ***B.*** Adenosine did not alter the I/O function representing excitability of the Schaffer collateral fibres. ***C.*** Adenosine inhibited synaptic transmission occurring at the CA3-CA1 synapse of the SC pathway. ***D.*** Adenosine showed no effect on postsynaptic (CA1 pyramidal cell) excitability. The apparent difference seen in Graph *D* is actually due to adenosine's preceding effect on synaptic transmission (Graph *C*).

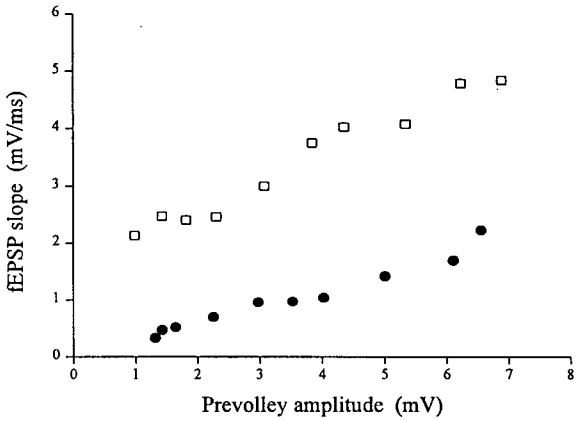
A.


Population spike amplitude (mV)
Stimulus intensity (V)

B.


Prevolley amplitude (mV)
Stimulus intensity (V)

C.


fEPSP slope (mV/ms)
Prevolley amplitude (mV)

D.

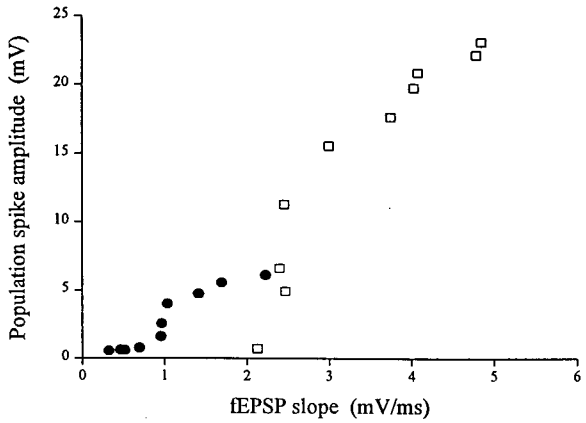
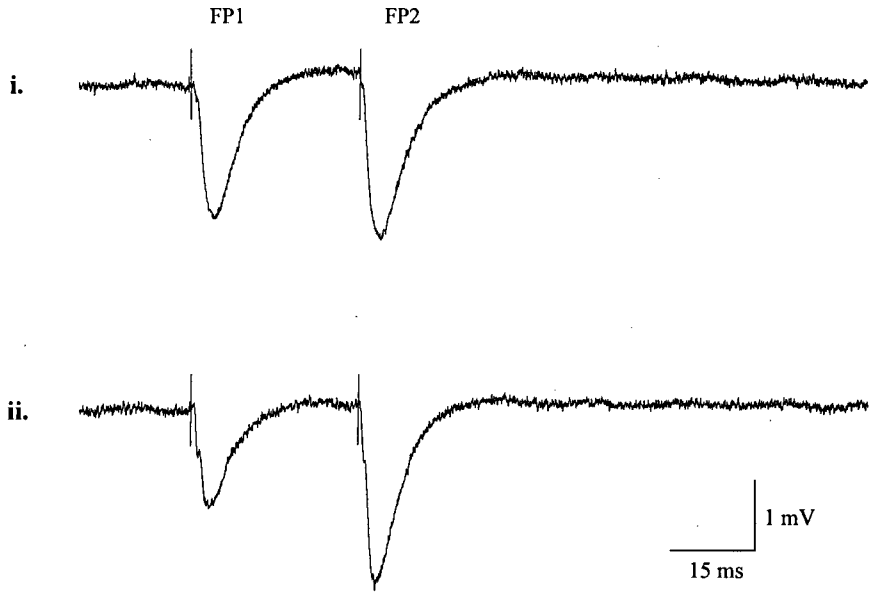

Population spike amplitude (mV)
fEPSP slope (mV/ms)

Figure 34. Effect of adenosine on the paired pulse facilitation ratio.

A. Paired stimulation (30 ms interstimulus interval) of the SC pathway evoked fEPSPs, recorded extracellularly from the dendritic region of CA1 pyramidal neurons. **i.** Paired stimulation in control (Mg^{2+}-containing) ACSF results in a slight facilitation of the second response (FP2) relative to the first response (FP1). **ii.** Adenosine (300 μM) caused an increase in the paired pulse facilitation ratio between the first (FP1) and second (FP2) responses. ***B.*** Averaged data from three experiments which shows the ratio between the amplitude of FP2 to that of FP1 over a range of interstimulus intervals. Error bars represent s.e.m. The first response (FP1) was normalized and the relative magnitude of the second response was then plotted. In all experiments, adenosine (black solid bars) significantly increased the ratio between FP2 and FP1 when compared to control responses (hatched bars). * $p < 0.05$, paired Student's *t*-test.

A.


FP1
FP2
i.
ii.
1 mV
15 ms

B.

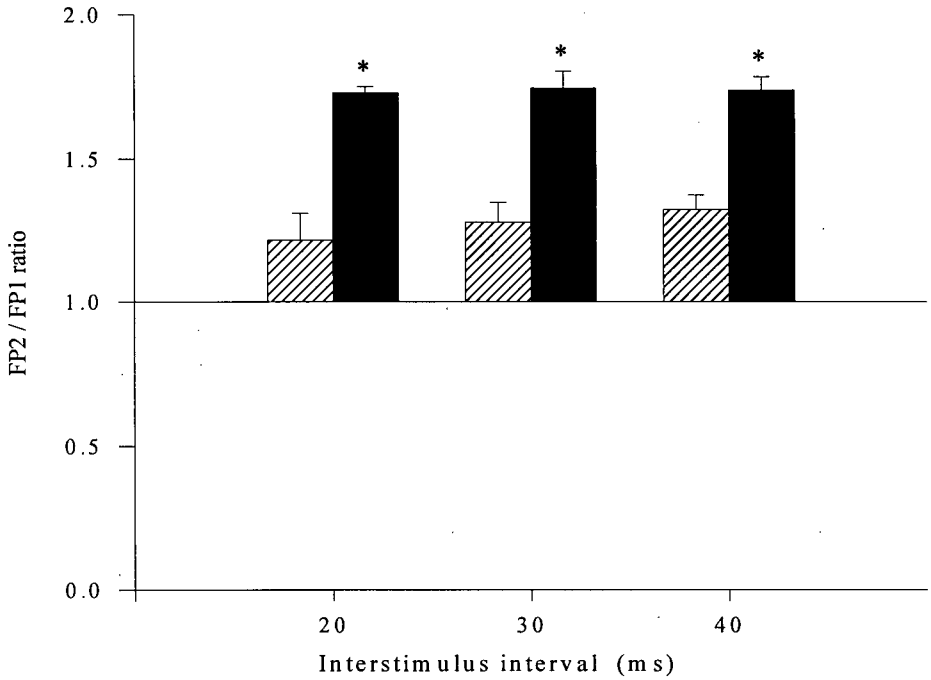
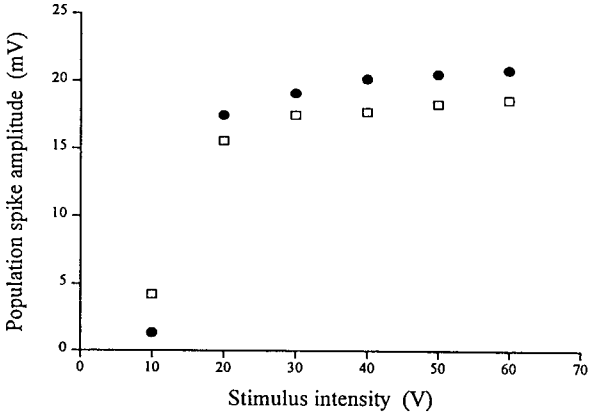

2.0
1.5
1.0
0.5
0.0
FP2 / FP1 ratio
*
*
*
20
30
40
Interstimulus interval (ms)

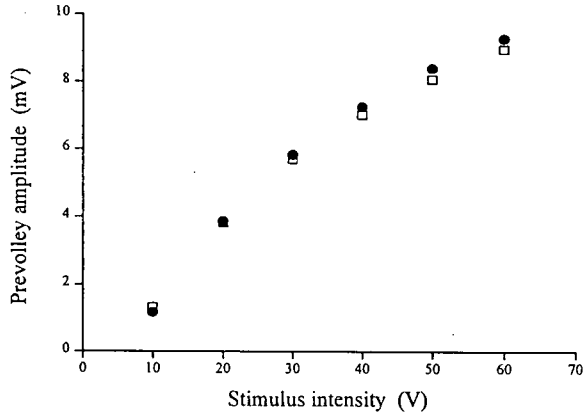
Figure 35. Results of a typical experiment showing the effects of DX on I/O functions along the CA3-CA1 pathway in the hippocampal slice.

A. The overall I/O function along the SC pathway was not affected by DX 10 μM (●) when compared to control (□) responses. ***B.*** Excitability of the Schaffer collaterals was not affected by DX. ***C.*** DX showed no effect on synaptic transmission at the CA3-CA1 synapse. ***D.*** Postsynaptic (CA1 pyramidal cell) excitability was not altered by DX.

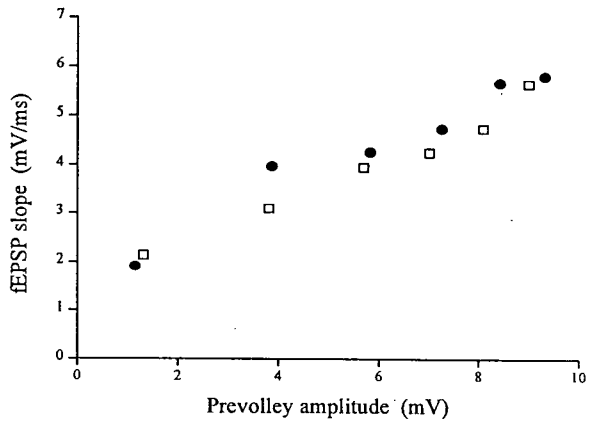
A.


Population spike amplitude (mV)
Stimulus intensity (V)

B.


Prevolley amplitude (mV)
Stimulus intensity (V)

C.


fEPSP slope (mV/ms)
Prevolley amplitude (mV)

D.

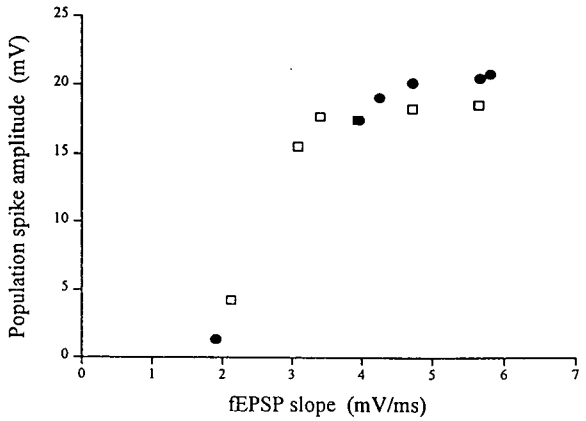
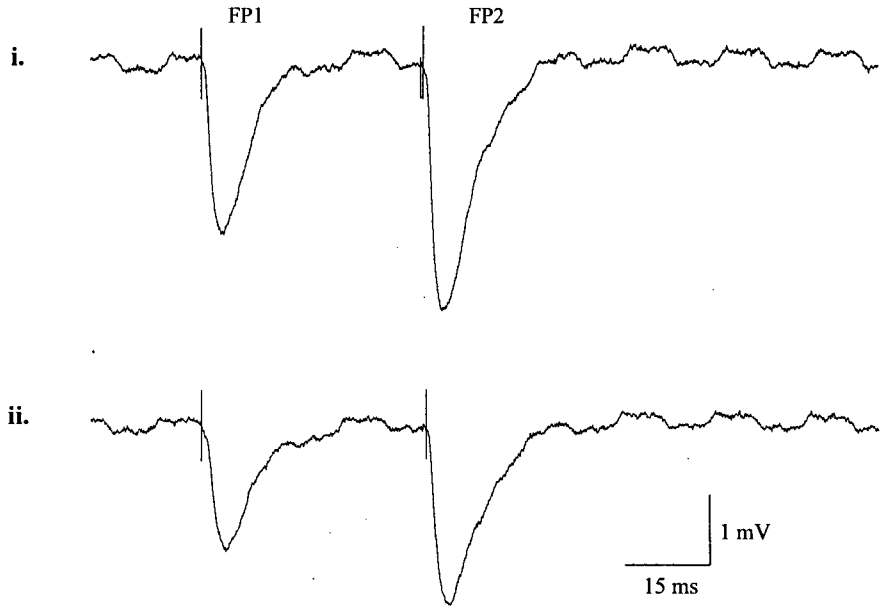

Population spike amplitude (mV)
fEPSP slope (mV/ms)

Figure 36. DX did not increase the paired pulse facilitation ratio.

A. Paired stimuli (40 ms interstimulus interval) of the SC pathway evoked fEPSPs, recorded extracellularly from the dendritic region of CA1 pyramidal neurons. **i.** Paired stimulation in control (Mg^{2+}-containing) ACSF results in a slight facilitation of the second response (FP2) relative to the first response (FP1). **ii.** DX (10 μM) did not significantly alter the ratio between the amplitudes of FP2 and FP1. ***B.*** Averaged data from four experiments showing the ratio between the amplitude of FP2 to that of FP1 over a range of interstimulus intervals. Error bars represent s.e.m. The first response (FP1) was normalized and the relative magnitude of the second response was then plotted. DX 10 μM (black solid bars) had no significant effect on the ratio between FP2 and FP1 when compared to control responses (hatched bars). * $p < 0.05$, paired Student's *t*-test. Similar results were observed with loperamide and ifenprodil (data not shown).

A.


FP1
FP2
i.
ii.
1 mV
15 ms

B.

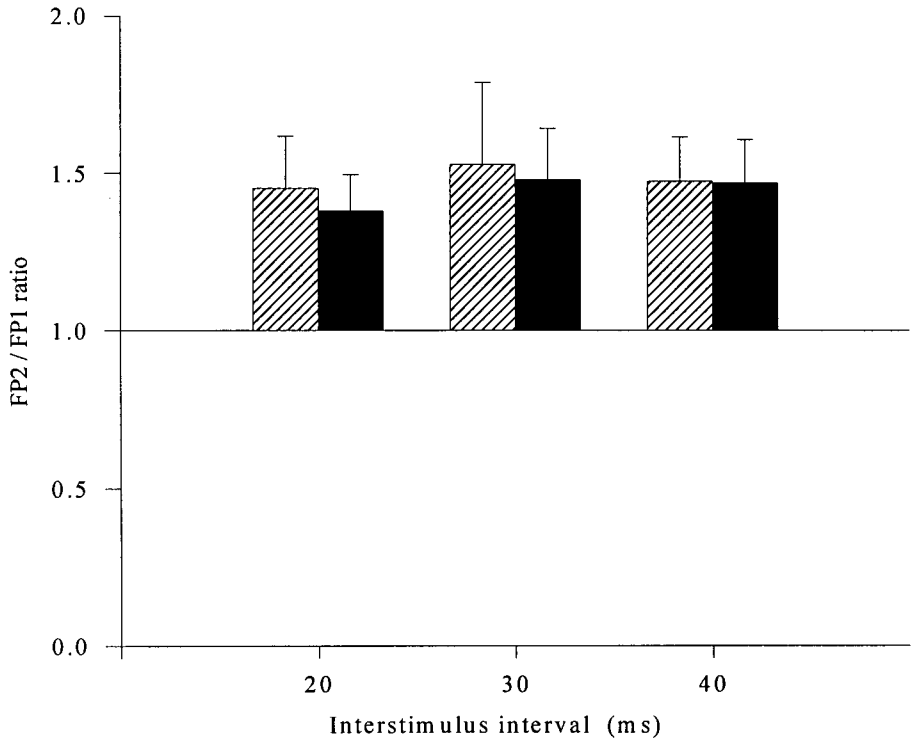
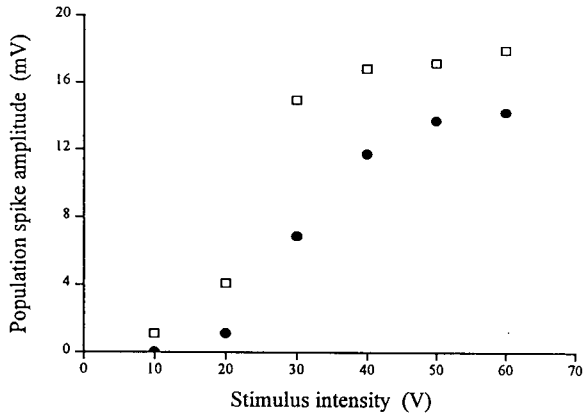

2.0
1.5
1.0
0.5
0.0
FP2 / FP1 ratio
20
30
40
Interstimulus interval (ms)

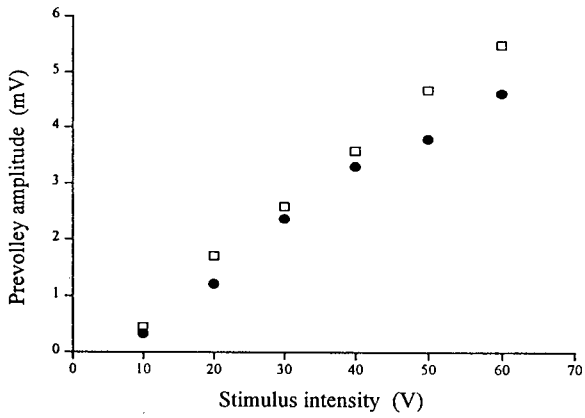
Figure 37. Results of a typical experiment showing the effects of caramiphen (CM) on I/O functions along the CA3-CA1 pathway in the hippocampal slice.

A. In the presence of 100 μM CM (●) an inhibitory effect on the overall I/O function of the SC pathway was observed when compared to control (□) responses. ***B.*** CM did not alter the excitability of the Schaffer collateral fibres. ***C.*** CM inhibited the SC pathway at the point of CA3-CA1 synaptic transmission. ***D.*** CM had no effect on postsynaptic (CA1 pyramidal cell) excitability. The apparent difference seen in Graph *D* is actually due to CM's preceding effect on synaptic transmission (Graph *C*).

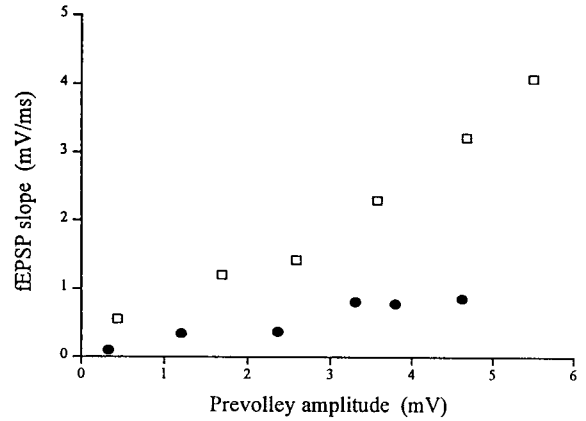
A.


Population spike amplitude (mV)
Stimulus intensity (V)

B.


Prevolley amplitude (mV)
Stimulus intensity (V)

C.


fEPSP slope (mV/ms)
Prevolley amplitude (mV)

D.

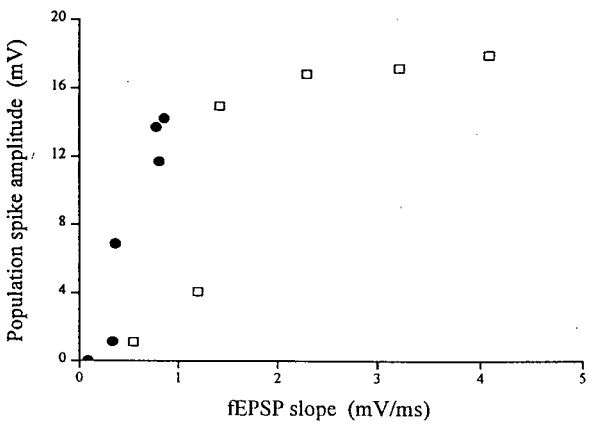
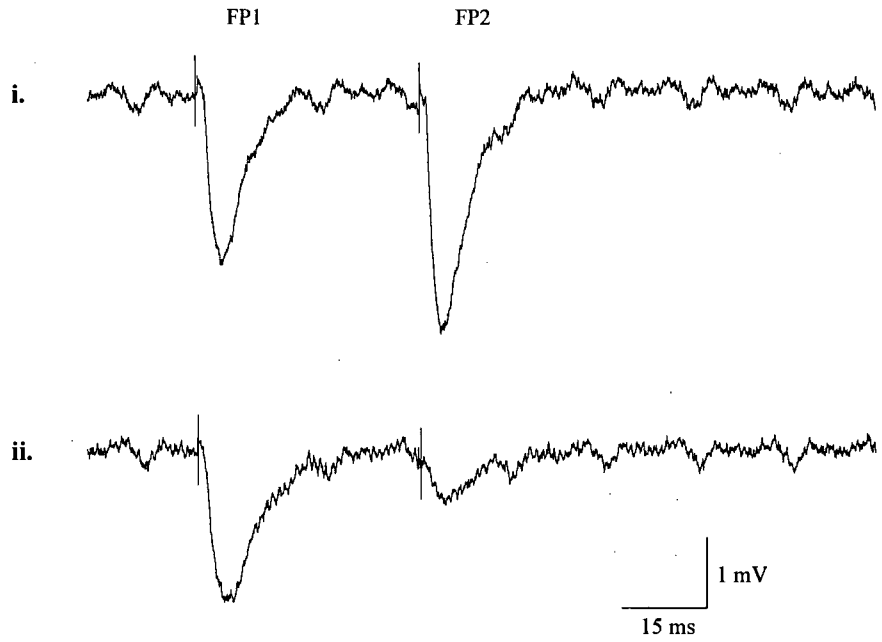

Population spike amplitude (mV)
fEPSP slope (mV/ms)

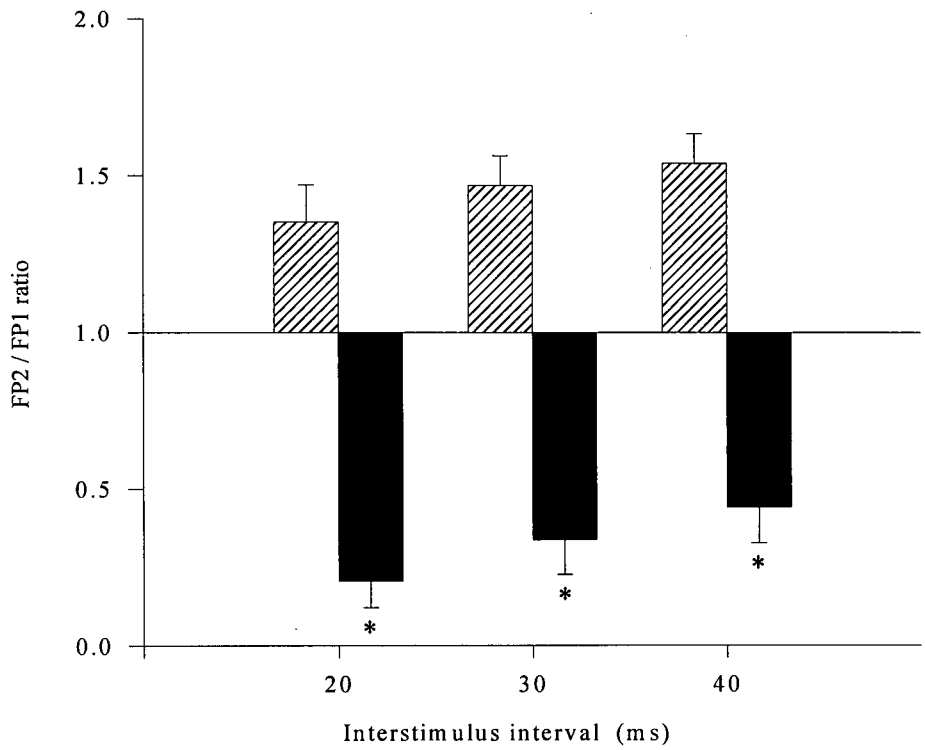
Figure 38. CM transformed paired pulse facilitation into paired pulse inhibition.

A. Paired stimuli (interstimulus interval 40 ms) of the SC pathway evoked fEPSPs, recorded extracellularly from the dendritic region of CA1 pyramidal neurons. **i.** Paired stimulation in control (Mg^{2+}-containing) ACSF results in a facilitation of the second response (FP2) relative to the first response (FP1). **ii.** CM (100 μM) decreased markedly the amplitude of the second response, resulting in a paired pulse inhibition of FP2 relative to FP1. ***B.*** Averaged data from four experiments showing the ratio between the amplitude of FP2 to that of FP1 over a range of interstimulus intervals. Error bars represent s.e.m. The first response (FP1) was normalized and the relative magnitude of the second response was then plotted. CM (black solid bars) significantly altered the FP2/FP1 ratio seen in control (hatched bars), transforming paired pulse facilitation to paired pulse inhibition. This resulted in a FP2/FP1 ratio below 1, as indicated by the black solid bars. * $p < 0.05$, paired Student's *t*-test.

A.


FP1
FP2
i.
ii.
1 mV
15 ms

B.


2.0
1.5
1.0
0.5
0.0
FP2 / FP1 ratio
20
30
40
Interstimulus interval (ms)
*
*
*

DISCUSSION

The compounds tested in this study all demonstrated concentration-dependent anticonvulsant activity against both evoked and spontaneous Mg^{2+}-free epileptiform activity. Although structurally dissimilar, this group of compounds all (apart from DX and with the possible exception of loperamide, which has not been examined) possess high (nM) affinity for sigma binding sites found in the brain. It has been suggested previously that these high-affinity σ receptors mediate, at least in part, the antiepileptiform (*e.g.* Aram *et al.*, 1989; Apland and Braitman, 1990; Pontecorvo *et al.*, 1991) and neuroprotective activities (*e.g.* Rao *et al.*, 1990; Pontecorvo *et al.*, 1991; DeCoster *et al.*, 1995; Yamamoto *et al.*, 1995) of high-affinity σ site ligands. However, results of the present study demonstrate that the anticonvulsant activity of the high-affinity σ site ligands examined is observed at micromolar concentrations. The present results therefore cast considerable doubt on the belief, widely held in the literature, that the anticonvulsant efficacy of high-affinity σ site ligands is mediated specifically by high-affinity σ binding sites.

If high-affinity σ binding sites do not mediate the anticonvulsant activity observed with μM concentrations of high-affinity σ ligands, what site(s) might be involved? Previously, it has been shown that a variety of high-affinity σ ligands (including many of those examined in the present study) are able to inhibit K^+-stimulated efflux of ^{86}Rb from cortical synaptosomes when applied at μM concentrations (Fletcher *et al.*, 1989). However, K^+ channel block is associated with the development of convulsive activity (see Introduction) and, indeed, K^+ channel openers have been shown to possess anticonvulsant activity (Gandolfo *et al.*, 1989).

Rather, the anticonvulsant activity of μM concentrations of high-affinity σ site ligands might reflect their ability to inhibit NMDA receptor-mediated events and/or HVA Ca^{2+} channels, effects which are only observed at micromolar concentrations (Church and Fletcher, 1995; Fletcher *et al.*, 1995). The latter observations, taken together with the well documented anticonvulsant activity of selective NMDA receptor antagonists and HVA Ca^{2+} channel blockers (see Introduction), would suggest that the anticonvulsant actions of the σ receptor ligands tested in the present study should not be ascribed to their high-affinity (nM) σ site binding, but rather to their blocking actions observed at micromolar concentrations at the NMDA receptor-channel complex and/or HVA Ca^{2+} channels.

i) Loperamide

Loperamide suppressed Mg^{2+}-free epileptiform activity in a concentration-dependent and reversible manner with an IC_{50} value for inhibition of evoked epileptiform activity of 28 μM. This is the first description of the anticonvulsant efficacy of loperamide. Loperamide, an anti-diarrhoeal compound, is known to mediate its anti-diarrhoeal effects via blockade of voltage activated Ca^{2+} channels (Reynolds *et al.*, 1984). Church *et al.* (1994a) recently demonstrated that loperamide also blocks multiple subtypes of neuronal HVA Ca^{2+} channels; low micromolar concentrations were shown to block high $[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ (IC_{50} = 0.9 μM) and whole-cell barium currents (IC_{50} = 2.5 μM) in cultured hippocampal pyramidal neurons. In the same study, loperamide also antagonized NMDA-evoked currents, although much higher concentrations were required (IC_{50} = 73 μM).

In the present study, if loperamide was exhibiting its anticonvulsant effects through blockade of HVA Ca^{2+} channels, it would be expected to show an anticonvulsant IC_{50} value in a range similar to that seen previously for selective HVA Ca^{2+} channel blockade. However, loperamide's anticonvulsant IC_{50} value determined in the current investigations (28 μM) lies above that required for effective HVA Ca^{2+} channel block (~ 1.7 μM; see Table 1). As inhibitory synaptic transmission in the hippocampus is known to remain intact in the Mg^{2+}-free epileptiform model, the possibility exists that loperamide was inhibiting presynaptic HVA Ca^{2+} channels responsible for neurotransmitter release at both excitatory (glutamatergic) *and* inhibitory (GABAergic) synapses resulting in at least a partial attenuation of any anticonvulsant effects consequent upon a decreased release of glutamate and/or a decreased influx of Ca^{2+} postsynaptically. Accordingly, higher concentrations may be required for effective anticonvulsant activity than those which are required for selective HVA Ca^{2+} channel blockade. Indeed, verapamil, which possesses a similar spectrum of activity to loperamide at HVA Ca^{2+} channels, has been shown to block both the release of glutamate from cerebellar slices (Barnes and Davies, 1988; Dickie and Davies, 1992) and depolarization-evoked release of GABA from rat hippocampal synaptosomes (Sitges and Reyes, 1995). Furthermore, recent *in vitro* studies have found that the HVA Ca^{2+} channel types responsible for both excitatory (glutamate) and inhibitory (GABA) neurotransmitter release at the CA3-CA1 synapse are pharmacologically similar (Horne and Kemp, 1991; Turner *et al.*, 1992; Burke *et al.*, 1993; Newcomb and Palma, 1994; see also Thate and Mayer, 1989). Additionally, Newcomb and Palma (1994) recently demonstrated *in vivo* that hippocampal glutamate and GABA release was affected in a nearly identical fashion by a number of ω-conopeptides with selective

activity at pre-synaptic HVA Ca^{2+} channel subtypes (see also Gaur *et al.*, 1994). To appreciate the complexity of the situation, the latter studies should however be compared with those of Potier *et al.* (1993) who found that ω-CgTx-GVIA blocked inhibitory synaptic transmission in the CA1 region of the rat hippocampus whilst reducing only partially evoked EPSPs (see also Ohno-Shosaku *et al.*, 1994).

The results of these studies might call into question whether blockade of presynaptic HVA Ca^{2+} channels responsible for neurotransmitter release does indeed represent a viable therapeutic target in seizure disorders. Various lines of evidence do however support the possible utility of presynaptic HVA Ca^{2+} channel blockade in anticonvulsant therapy. *First*, selective blockers of P- and Q-type Ca^{2+} channels, which are involved in neurotransmitter release, show anticonvulsant activity in the rat cortical wedge preparation (Robichaud *et al.*, 1994). *Second*, presently available anticonvulsant agents such as phenytoin, carbamazepine, phenobarbital and lamotrigine are believed to act, at least in part, by inhibiting (excitatory) neurotransmitter release (Crowder and Bradford, 1987; Upton, 1994). *Third*, adenosine, which has been suggested to act as the body's endogenous anticonvulsant (Dragunow, 1991), acts via presynaptic A1 receptors to inhibit evoked excitatory synaptic transmission primarily by reducing presynaptic Ca^{2+} influx through ω-CgTx-GVIA-sensitive (N-type) Ca^{2+} channels (Yawo and Chuhma, 1993; Wu and Saggau, 1994a; but see Horne and Kemp, 1991). *Fourth*, verapamil and flunarizine are able to inhibit responses mediated by Ca^{2+} flux through DHP-sensitive and -insensitive Ca^{2+} channels, including synaptosomal Ca^{2+} influx and DHP-insensitive Ca^{2+}-dependent release of endogenous glutamate (Barnes and Davies, 1988; Mangano *et al.*, 1991; Bowman *et al.*, 1993; Cousin *et al.*, 1993). Both compounds have

demonstrated anticonvulsant activity in a variety of animal seizure models (Wauquier *et al.*, 1985; Bingmann and Speckmann, 1989; DeSarro *et al.*, 1990; Czuczwar *et al.*, 1992), while flunarizine has been employed in humans as an adjuvant in the treatment of seizure disorders that are incompletely controlled by conventional anticonvulsants (Binnie *et al.*, 1985). *Fifth*, agonists of presynaptic mGluRs, which act as autoreceptors to decrease glutamate release, have demonstrated anticonvulsant activity both *in vitro* and *in vivo* (Sheardown, 1992; Burke and Hablitz, 1994; Thomsen *et al.*, 1994; Attwell *et al.*, 1995).

Loperamide inhibited both evoked and spontaneous epileptiform activity; however, a more consistent and potent effect was observed on spontaneous activity originating from the CA3 region than on the activity evoked in area CA1 by stimulation of the SC pathway. As loperamide is known to possess potent HVA Ca^{2+} channel blocking actions, extensive Ca^{2+} channel mediated currents in the CA3 region may explain its preferential effect on spontaneous epileptiform activity which arises in CA3 pyramidal cells. For example, CA3 pyramidal neurons possess more well developed VSCC currents in comparison to pyramidal neurons in the CA1 region (Wong and Prince, 1978). Moreover, ω-Aga-IVA-sensitive (P-type) Ca^{2+} channels, which contribute significantly to glutamate release, have recently been localized on the presynaptic terminals of CA3 pyramidal neurons, in addition to their established presence on CA3 pyramidal cell bodies (Mintz *et al.*, 1992).

As loperamide is known to possess broad-spectrum HVA Ca^{2+} channel blocking activity, both I/O functions and PPF ratios were examined in its presence, in order to further assess its locus of action and, more specifically, to examine whether presynaptic HVA Ca^{2+} channel blockade was contributing to its anticonvulsant actions. Under normal physiological

conditions and at most excitatory synapses (including the CA3-CA1 synapse), paired subthreshold stimuli (20-70 ms ISI), will evoke two dendritic fEPSPs where there is a facilitation of the amplitude of the second response when compared to that of the first (Harris and Cotman, 1983; Dunwiddie and Haas, 1985; Zucker, 1989). An example of paired pulse facilitation (PPF) in the present experiments is seen in Figure 38*A*i. This type of short term plasticity can be explained by the residual Ca^{2+} theory as proposed by Katz and Miledi (1968) and reflects the persistence of Ca^{2+} in the presynaptic terminal following an initial stimulus and a subsequent facilitated neurotransmitter release with a second stimulus. Originally stemming from observations at the frog neuromuscular junction, the residual Ca^{2+} theory has received recent support from Ca^{2+} imaging experiments in the CA1 hippocampal region, which demonstrated that there is indeed a transient increase in the presynaptic residual calcium level following a first stimulus (Wu and Saggau, 1994b). Furthermore, it has now been well established that compounds known to inhibit neurotransmitter release display an increased PPF ratio when compared to control ratios (*e.g.* Harris and Cotman, 1983; Dunwiddie and Haas, 1985; Kahle and Cotman, 1993; Simmons *et al.*, 1994). This effect has been observed in the CA1 hippocampal region not only with endogenous compounds such as adenosine (Dunwiddie and Haas, 1985) and spermine (Ferchmin *et al.*, 1995), but also with specific inhibitors of N- and Q-type Ca^{2+} channels, which in turn are known to be involved in neurotransmitter release (Wheeler *et al.*, 1994b; Ferchmin *et al.*, 1995). However, despite previous evidence for HVA Ca^{2+} channel blocking actions, loperamide (and ifenprodil; see below) demonstrated no effect on the I/O functions or PPF ratios, reminiscent of the results observed with the selective NMDA antagonist, dextrorphan (see Figs. 35 and 36).

Loperamide's lack of effect on the PPF ratio indicate that it does not possess activity at those presynaptic HVA Ca^{2+} channels involved in glutamate release. However, it was surprising that no effect was observed on the synaptic transfer I/O function in the presence of loperamide, as one would assume that if not presynaptic in its action, loperamide must mediate its inhibitory actions postsynaptically. However, although HVA Ca^{2+} channels have been located postsynaptically on CA1 dendrites (Jaffe *et al.*, 1992; Regehr and Tank, 1992) their role, if any, in the propagation of synaptic potentials under normal conditions remains uncertain (Luebke *et al.*, 1993). Therefore, if loperamide was mediating its anticonvulsant actions via blockade of these postsynaptic Ca^{2+} channels, it is possible that its effect would only be observed under conditions (such as seizure activity), when these channels are known to contribute to postsynaptic depolarization. The results observed in the I/O and PPF experiments, which were conducted in the presence of external Mg^{2+} (*i.e.* under non-epileptiform conditions) therefore suggest that loperamide's anticonvulsant activity is mediated solely via blockade of postsynaptic HVA Ca^{2+} channels. Interestingly, a similar absence of effect on non-epileptiform synaptic activity has also been observed both *in vitro* and *in vivo* with the organic Ca^{2+} channel blockers verapamil and flunarizine. In fact, this lack of effect on non-epileptiform activity, which indicates that normal information processing is unaffected, may actually be responsible in part, for the paucity of central side effects observed upon the administration of these compounds at therapeutically-useful anticonvulsant doses (Pohl *et al.*, 1992). Loperamide's similar absence of effect on normal synaptic transmission observed in this study suggests that it too might demonstrate therapeutic activity relatively free of neurobehavioural side effects.

ii) Caramiphen, carbetapentane and opipramol

The non-opioid antitussives caramiphen (CM) and carbetapentane (CBT) exhibited concentration-dependent anticonvulsant actions against epileptiform activity evoked by stimulation of the SC pathway with IC_{50} values of 46 and 38 μM, respectively. These values are in close agreement with those observed by other investigators (Aram *et al.*, 1989; Apland and Braitman, 1990). In all experiments where evoked epileptiform activity was affected, CM and CBT also reduced the spontaneous epileptiform activity originating from the CA3 region. CM demonstrated a particularly potent effect on spontaneous epileptiform activity, abolishing it even at the lowest concentration (20 μM) tested.

CM and CBT are known to possess blocking activity at multiple subtypes of HVA Ca^{2+} channels. For example, Church and Fletcher (1995) recently demonstrated that CM and CBT block whole-cell barium currents in cultured hippocampal neurons with IC_{50} values of 47 and 40 μM, respectively. Both compounds also inhibit K^+-stimulated release of endogenous glutamate (*e.g.* IC_{50} value for CBT = 40 μM; Annels *et al.*, 1991). Interestingly, the concentration of CBT required for neuroprotection (IC_{50} = 46 μM for reduction of glutamate-induced injury in cultured rat cortical neurons; DeCoster *et al.*, 1995) is very similar to that required for anticonvulsant activity in the present study and has also been ascribed to CBT's blockade of HVA Ca^{2+} channels. Indeed, activity at HVA Ca^{2+} channels could well account for CM's potent effect on spontaneous epileptiform activity originating from the CA3 region, whose pyramidal neurons are known to have more well-developed VSCC currents than do CA1 pyramidal neurons (Wong and Prince, 1978; see above).

Compounds such as CM and CBT (and opipramol; see below) theoretically have the potential to act synergistically by blocking both the NMDA receptor and HVA Ca^{2+} channels. Acting in this manner, these compounds might demonstrate effective anticonvulsant IC_{50} concentrations below those required for selective blockade of either system independently. In such a way, lower doses of these compounds would be required to attain the desired therapeutic effect (*i.e.* anticonvulsant activity) than compounds which act at one system alone. However, the IC_{50} values for the anticonvulsant activities of both CM and CBT observed in the present study lie in close proximity to the concentration range required for selective HVA Ca^{2+} channel blockade (see Table 1), and would suggest that their anticonvulsant effects are mediated almost entirely by HVA Ca^{2+} channel blockade, with NMDA receptor antagonism contributing little (if any) to their therapeutic properties.

CM was employed in the I/O experiments as an agent representative of dual action compounds which block HVA Ca^{2+} channels and which also possess some NMDA receptor antagonist activity. Evidence obtained from the I/O experiments suggested that CM was mediating its anticonvulsant actions at the point of synaptic transfer (see Fig. 38). Thus, PPF experiments were then performed to determine whether blockade of pre- or postsynaptic HVA Ca^{2+} channels was responsible for the decrease in synaptic efficacy observed in the I/O analysis. Given the fact that CM is known to block HVA Ca^{2+} channels involved in neurotransmitter release, it was anticipated that CM would increase the PPF ratio, in a manner similar to that observed with adenosine (see Fig. 34). However, CM significantly decreased the PPF ratio, to such an extent that a paired pulse inhibition (PPI) was observed, with a significant decrease of the amplitude of the second response relative to the amplitude of the first response (see Fig. 38).

Although initially surprising, a number of explanations could account for CM's apparently paradoxical effect on the PPF ratio. Firstly, intracellular recordings have revealed that electrical stimulation of the SC pathway evokes an EPSP followed by an IPSP. Therefore in the PPF experiments (20 - 40 ms ISI), the reduction in the amplitude of the second fEPSP in the presence of CM could reflect the ability of CM to potentiate the GABA-mediated IPSP following the first stimulus, which could then attenuate the EPSP resulting from the second stimulus, resulting in a PPI. In this regard, PPI has previously been observed with GABA-mimetics such as diazepam (Lee *et al.*, 1979) and pentobarbital (Ashton *et al.*, 1988), which are known to increase GABA-mediated inhibitory Cl^- currents. Unfortunately, there are no reports in the literature concerning the effects of CM on $GABA_A$ or $GABA_B$ receptor-mediated responses.

Alternatively, the effects of CM on inhibitory circuits within the hippocampus may represent a second possibility for the observed results of CM in the PPF experiments. CA3 afferents not only synapse directly on dendrites of CA1 pyramidal neurons, but also send off collateral branches which excite inhibitory interneurons. These inhibitory interneurons in turn synapse on CA1 pyramidal cell dendrites, thus mediating feed forward inhibition (Lacaille *et al.*, 1989; see Fig. 1*B*). PPF is a protocol designed to reflect monosynaptic events and is strictly homosynaptic (*i.e.* the facilitation is only observed at the synapse which is being stimulated; Creager *et al.*, 1980; Higgins and Stone, 1996). However, because the electrode used in the present experiments to stimulate the SC pathway was fairly coarse, both the axons which synapse directly onto CA1 pyramidal cells and their collaterals (which mediate inhibition) would inevitably have been recruited. The ramifications of this possible scenario and its ability to account for CM's PPI effect will be discussed further below.

Although the results of PPF experiments have largely been interpreted in terms of the effect of a given test compound on excitatory neurotransmitter release, the same concepts also hold true for neurotransmitter release at inhibitory terminals. For example, PPF of IPSCs (40 ms ISI) has been demonstrated in CA1 inhibitory interneurons by Roepstorff and Lambert (1994). Furthermore, under conditions which decrease the probability of neurotransmitter (GABA) release (*e.g.* raising $[Mg^{2+}]_o$), there is a marked increase in the PPF of IPSCs (20 - 50 ms ISI; Lambert and Wilson, 1994). This facilitation results in a potentiated IPSP following the second stimulus which would serve to decrease the amplitude of the second fEPSP in relation to the first fEPSP. This is exactly what was observed with CM in the present study.

If the above scenario is true, one has to postulate that CM, via blockade of presynaptic HVA Ca^{2+} channels, is able to inhibit both glutamate *and* GABA release (the PPI observed in this study suggests a preference for GABAergic terminals), and that its anticonvulsant activity is not dependent on the sum of these effects. In this regard, it is notable that both CM and adenosine had similar effects on I/O functions (both compounds mediating their inhibitory effects at the point of synaptic transfer), but opposite effects in the PPF experiments (adenosine increasing the PPF ratio and CM causing PPI). These contrasting results can be explained by the fact that adenosine has been shown to reduce glutamate release while, at the same time, having no effect on GABA release (Lambert and Teyler, 1991; Yoon and Rothman, 1991). Indeed, A1 receptors (which mediate the ability of adenosine to indirectly inhibit N-type Ca^{2+} channels) are present only on pyramidal neurons and excitatory terminals in the CA1 region; they are absent from inhibitory terminals (Lambert and Teyler, 1991; Thompson *et al.*, 1992). In contrast, CM exerts a direct action at HVA Ca^{2+} channels and might be expected to

affect the release of both excitatory (*i.e.* glutamate) and inhibitory (*i.e.* GABA) neurotransmitters.

Given that adenosine and CM demonstrate such contrasting effects on PPF ratios it seems remarkable that they would possess a common anticonvulsant action. A likely explanation is that adenosine's inhibitory actions are mediated solely by blockade of presynaptic HVA Ca^{2+} channels responsible for glutamate release. CM on the other hand, possesses blocking activity at both N- and L-type HVA Ca^{2+} channels (Church and Fletcher, 1995), and it is entirely plausible that CM's anticonvulsant actions arise from its blockade of postsynaptic (most likely L-type) HVA Ca^{2+} channels, while its presynaptic effects on both glutamate and GABA release serve only to nullify one another. If this indeed represents what is occurring, similar drugs should be developed which, like CM, can inhibit both postsynaptic HVA Ca^{2+} channels and reduce glutamate release, but which have no effect on GABA release. In conclusion, the results presented in this work indicate that CM's anticonvulsant activity is predominantly mediated by postsynaptic HVA Ca^{2+} channel blockade.

Opipramol was the third compound tested which displays HVA Ca^{2+} channel blocking actions with some NMDA receptor antagonism. Opipramol demonstrated anticonvulsant actions against Mg^{2+}-free evoked epileptiform activity (IC_{50} = 52 μM), a finding which is the first description of its anticonvulsant properties. Spontaneous epileptiform activity originating from the CA3 hippocampal region was also effectively decreased by opipramol.

Opipramol has previously been shown to possess neuroprotective activity *in vivo*; its activity in this regard however has been ascribed to its nanomolar affinity for high-affinity σ binding sites (Rao *et al.*, 1990). For example, Ferris *et al.* (1991) found that opipramol

displaced the archetypal high-affinity σ site ligand, [^{3}H](+)-3-PPP [R(+)3-(3-hydroxyphenyl)-N-(n-propyl)piperidine] from rat brain membranes with a K_i value of 6 nM. However, opipramol also possesses HVA Ca^{2+} channel and NMDA receptor blocking actions at μM concentrations (Fletcher *et al.*, 1995; see Table 1), actions which appear more likely to underlie its anticonvulsant effects, which were also observed at μM concentrations.

Opipramol's mechanism of NMDA receptor blockade has not been elucidated, although the absence of use- and voltage-dependency in opipramol's blocking actions would argue against open channel blockade (*e.g.* at the PCP or Mg^{2+} site), and Fletcher *et al.* (1995) have therefore proposed that a novel site on the NMDA receptor may mediate opipramol's NMDA antagonist actions.

Opipramol has also been shown to block HVA Ca^{2+} channels, again in the μM concentration range, an effect which may also contribute to its anticonvulsant activity (Church and Fletcher, 1995). However, since opipramol blocks both HVA Ca^{2+} channels and NMDA receptor-mediated currents it is difficult to assess by which mechanism(s) opipramol might be exerting its anticonvulsant actions. However, the effective concentration required for opipramol's anticonvulsant activity in the current investigation (IC_{50} = 52 μM) is *not* lower than that required for selective NMDA receptor or HVA Ca^{2+} channel blockade, but rather lies between them. This suggests that opipramol's inhibitory actions at NMDA and Ca^{2+} channels do not result in an additive anticonvulsant effect as was originally hypothesized. In a fashion similar to loperamide, opipramol is perhaps inhibiting a broad spectrum of HVA Ca^{2+} channels, possibly including those which mediate GABA release. This would create an attenuation of opipramol's anticonvulsant effects mediated by NMDA receptor and/or Ca^{2+}

channel blockade, and would result in a need for greater concentrations to produce equieffective anticonvulsant activity.

iii) Ifenprodil, dextromethorphan and 1,3-di(2-tolyl)guanidine

Ifenprodil suppressed epileptiform activity evoked by stimulation of the SC pathway during perfusion with Mg^{2+}-free medium with an IC_{50} value of 6 μM. Spontaneous epileptiform bursts were also effectively reduced by ifenprodil in a concentration-dependent manner. Ifenprodil has previously demonstrated anticonvulsant activity both *in vitro* and *in vivo,* activity which, as in the cases of CBT and CM, has been ascribed to its affinity for high-affinity σ binding sites (*e.g.* DeSarro and DeSarro, 1993). Thus, ifenprodil has been shown to displace [^{3}H](+)-3-PPP binding to rat brain homogenates with an IC_{50} value of 12 nM (Contreras *et al.*, 1990). In contrast, ifenprodil's anticonvulsant activity is associated with μM concentrations of the compound, concentrations at which ifenprodil is known to inhibit both NMDA receptor-mediated events and HVA Ca^{2+} channels.

As an antagonist of NMDA-evoked whole-cell currents in hippocampal neurons, ifenprodil was found to have an IC_{50} value of 0.8 μM (Fletcher *et al.*, 1995), which is in reasonable proximity to its IC_{50} value for anticonvulsant activity observed in this study (6 μM), suggesting that ifenprodil's anticonvulsant effects are mediated, at least in part, by NMDA receptor antagonism. With regard to ifenprodil's mechanism of blockade at the NMDA receptor, a large body of evidence suggests that it modulates NMDA receptor activation via the polyamine site on the NMDA receptor-channel complex (Carter *et al.*, 1989; Schoemaker *et al.*, 1990; Williams *et al.*, 1991), although not in a purely competitive fashion,

as recently indicated by Church *et al.* (1994b). Its NMDA receptor blocking action is not use- or voltage-dependent, which argues against an open channel block (Fletcher *et al.*, 1995). NMDA receptor subtype selectivity for the NMDAR1/NMDAR2B receptor type has also been observed as an aspect of ifenprodil's characteristics (Williams, 1993; Nicolas and Carter, 1994); it has been postulated that this selectivity may account for ifenprodil's relative lack of behavioural side effects (Nankai *et al.*, 1995).

In addition to NMDA receptor antagonism, ifenprodil also possesses HVA Ca^{2+} channel blocking activity. Thus, ifenprodil can block whole-cell barium currents in voltage-clamped cultured hippocampal neurons (IC_{50} = 18 μM; Church *et al.*, 1994b), high K^+-evoked rises in $[Ca^{2+}]_i$ in cultured hippocampal neurons (IC_{50} values for DHP-sensitive and DHP-insensitive components = 17 μM and 13 μM, respectively; Church *et al.*, 1994b) and K^+-stimulated release of glutamate in rat striatal slices (IC_{50} = 35 μM; Ellis and Davies, 1994; see also Mangano *et al.*, 1991). These concentrations are again reasonably similar to the concentrations of ifenprodil required for effective anticonvulsant activity (IC_{50} = 6 μM). Thus, the anticonvulsant actions of ifenprodil are likely mediated, at least in part, by both NMDA receptor antagonism and blockade of HVA Ca^{2+} channels. Nevertheless, ifenprodil also has effects on numerous other neurotransmitter systems, including antagonism of serotonin receptors (McCool and Lovinger, 1995) and inhibition of dopamine uptake (Izenwasser *et al.*, 1993), effects which may also contribute to its profile of anticonvulsant activity. Most recently, Stoehr *et al.* (1996) reported that ifenprodil can block voltage-activated Na^+ channels in a voltage dependent manner (IC_{50} = 30 μM) and have suggested that this action may also be involved in its anticonvulsant activity.

Interestingly, the ability of ifenprodil to block both NMDA receptor-mediated events and HVA Ca^{2+} channels is shared by an analog of ifenprodil, eliprodil. Although the anticonvulsant properties of eliprodil have not been investigated, it is interesting to note that antagonism at both NMDA receptors and at HVA Ca^{2+} channels have been suggested to underlie the known neuroprotective actions of eliprodil (Biton *et al.*, 1994).

In similar fashion to loperamide, ifenprodil failed to show any effect on the I/O functions and PPF ratio. Although inconclusive, ifenprodil's absence of effect on the I/O functions suggests that its anticonvulsant activity is mediated primarily by blockade of postsynaptic HVA Ca^{2+} channels (see loperamide) and/or NMDA receptor antagonism (see DX below), while the PPF result indicates that blockade of presynaptic HVA Ca^{2+} channels is unlikely to contribute to ifenprodil's anticonvulsant actions (*e.g.* see the discussion concerning loperamide above).

DXM demonstrated potent anticonvulsant activity against evoked Mg^{2+}-free epileptiform activity, and in the same concentration range was able to reduce both the amplitude and frequency of Mg^{2+}-free induced spontaneous epileptiform bursts. The IC_{50} value for DXM against evoked epileptiform activity determined in this investigation was 10 μM, which is in close agreement with the value determined by Apland and Braitman (1990) against Mg^{2+}-free epileptiform activity in guinea pig hippocampal slices (EC_{50} = 18 μM) and by Aram *et al.* (1989) in rat neocortical slices (IC_{50} = 10 - 15 μM). *In vivo*, DXM is also well established as an effective anticonvulsant agent (*e.g.* Leander *et al.*, 1988; Löscher and Hönack, 1993; De Sarro and De Sarro, 1993).

It has been suggested that high-affinity (nM) binding to σ receptor site(s) mediates DXM's anticonvulsant actions (Klein and Musacchio, 1989; Musacchio *et al.*, 1989). However, similar to many of the compounds tested in the present study, there is no apparent correlation between DXM's nanomolar affinity for these sites and the micromolar concentrations required for effective anticonvulsant activity. On the other hand, μM concentrations of DXM are known to exhibit NMDA antagonist activity. Thus, DXM's IC_{50} value for inhibition of [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine (TCP; a PCP analog) binding is 1.76 μM (Yamamoto *et al.*, 1995), exactly the same value found by Fletcher *et al.* (1995) for inhibition of NMDA-evoked currents in voltage-clamped mouse hippocampal neurons (see Table 1). Interestingly, the potency of DXM as an NMDA receptor antagonist also agrees well with its neuroprotective potency against glutamate toxicity in cultured rat cortical neurons (IC_{50} = 3 μM; DeCoster *et al.*, 1995).

DXM also possesses HVA Ca^{2+} channel blocking properties, but at concentrations ranging from 50-80 μM (Netzer *et al.*, 1993; Church and Fletcher, 1995; also see Table 1), values which are considerably in excess of the concentrations required for anticonvulsant activity. In addition, DXM is able to block K^+-stimulated release of glutamate from rabbit hippocampal slices but once again this effect is observed at concentrations (IC_{50} = 130 μM; Annels *et al.*, 1991) greatly in excess of those associated with anticonvulsant activity (this study; also Aram *et al.*, 1989; Apland and Braitman, 1990). These observations suggest that HVA Ca^{2+} channel blockade is unlikely to mediate directly DXM's anticonvulsant properties. Rather, DXM's anticonvulsant properties appear to reflect NMDA receptor antagonism. However, in addition to NMDA receptor blocking actions, Na^+ channel blockade might also

contribute to DXM's anticonvulsant properties, as DXM has been found to block voltage dependent Na^+ channels at micromolar concentrations (IC_{50} = 78 μM; Netzer *et al.*, 1993).

DTG inhibited both evoked and spontaneous Mg^{2+}-free epileptiform activity in a concentration-dependent manner, the IC_{50} value for reduction of evoked epileptiform activity being 15 μM. DTG is a selective σ receptor ligand at low (nM) concentrations (see Quirion *et al.*, 1992), but at higher (μM) concentrations it can interact with the NMDA receptor-channel complex (Connick *et al.*, 1992; Monnet *et al.*, 1992; Fletcher *et al.*, 1993). For example, Keana *et al.* (1989) determined that DTG displaces [^{3}H]-TCP and (+)-[^{3}H]MK-801 from the PCP receptor in brain membrane preparations with IC_{50} values of 8 and 11 μM, respectively. These concentrations are much greater than those observed for DTG's binding to high-affinity σ sites (IC_{50} = 53 nM for displacement of [^{3}H](+)-3-PPP binding from guinea pig membranes; Weber *et al.*, 1986), but very similar to the concentrations required for neuroprotection (Keana *et al.*, 1989; Pontecorvo *et al.*, 1991; Long *et al.*, 1992; Shalaby *et al.*, 1992) and anticonvulsant activity (this study; also Aram *et al.*, 1989). The IC_{50} value determined for anticonvulsant activity in the present study also agrees well with the concentration required for reduction of NMDA-evoked whole-cell currents (IC_{50} = 37 μM; Fletcher *et al.*, 1995). Although DTG is able to block whole-cell barium currents, the concentration required (IC_{50} = 200μM; Table 1) is well above that required for its anticonvulsant activity (this study; also Aram *et al.*, 1989). This indicates that DTG's anticonvulsant properties arise primarily as a result of its NMDA receptor antagonist actions.

Although DTG (and DX; see below) were anticonvulsant in their overall effect, low concentrations of these compounds occasionally increased the size of the first (and sometimes

subsequent) population spikes following SC stimulation. This anomaly has been observed specifically with DX by Aryanpur *et al.* (1990), while a similar finding was seen with DTG by Bergeron *et al.* (1995). A similar 'low-dose' enhancement of epileptiform activity has been observed in the presence of various other NMDA receptor antagonists (Dingledine *et al.*, 1986; King and Dingledine, 1986; Neuman *et al.*, 1988; Cole *et al.*, 1989). In the case of DTG, it has been suggested that although DTG possesses NMDA receptor-mediated anticonvulsant actions, the potentiating effect on the primary population spike(s) may actually be mediated by the high-affinity σ binding site (Aryanpur *et al.*, 1990; Bergeron *et al.*, 1995)

In summary, the anticonvulsant IC_{50} values obtained in this study for such 'dual action' compounds as opipramol, ifenprodil, DXM and DTG seem to suggest that NMDA receptor blockade is not acting in concert with HVA Ca^{2+} channel blocking activity to produce an additive anticonvulsant effect. This would indicate, perhaps, that their HVA Ca^{2+} channel blocking actions are *too* broad, and inhibit not only excitatory neurotransmission but also the release of inhibitory neurotransmitters such as GABA. The situation is further complicated by effects these compounds might exert on other neurotransmitter systems. For example, Thate and Meyer (1989) found that ω-CgTx-GVIA can reduce not only the release of GABA directly, but can have an additional inhibitory effect on somatostatin neurons which are known to depress the release of GABA. Furthermore, N-type Ca^{2+} channel blockers also reduce the release of noradrenaline (Dooley *et al.*, 1987 & 1988; Sabrià *et al.*, 1995) and serotonin (Pullar and Findlay, 1992), which can result in both pro- and anticonvulsant effects. Thus, proconvulsant influences would attenuate the anticonvulsant activity mediated via NMDA

receptor antagonism and/or postsynaptic HVA Ca^{2+} channel blockade, resulting in a need for greater concentrations to achieve anticonvulsant efficacy.

iv) Dextrorphan

Dextrorphan (DX) demonstrated potent anticonvulsant effects on both evoked and spontaneous epileptiform activity, with an IC_{50} value against evoked epileptiform activity of 2 μM. Spontaneous epileptiform activity was also diminished in a concentration-dependent manner following exposure to DX. DX, a potent anticonvulsant agent both *in vitro* and *in vivo* (*e.g.* Leander *et al.*, 1988; Tortella *et al.*, 1988; Chapman and Meldrum, 1989; Cole *et al.*, 1989), is known to mediate its anticonvulsant effects via blockade of the NMDA receptor. It does not interact with high-affinity σ sites (Franklin and Murray, 1992), HVA Ca^{2+} channels (Carpenter *et al.*, 1988; Jaffe *et al.*, 1992) or non-NMDA receptors (Church *et al.*, 1985). The concentrations required for anticonvulsant activity in this study (see also Aram *et al.*, 1989) approximate closely those required for blockade of NMDA receptor-evoked responses in both rat cortical slices (IC_{50} = 3 μM; Aram *et al.*, 1989) and hippocampal pyramidal neurons (IC_{50} = 0.35 μM; see Church *et al.*, 1989), thereby providing further support for the NMDA receptor-mediated anticonvulsant action of DX. Moreover, DX's lack of affinity for σ sites provides further evidence that high-affinity σ binding sites do no mediate anticonvulsant activity.

In the present study, DX's absence of effect on I/O functions and PPF ratios support the possibility that DX acts solely at the NMDA receptor-channel complex. Interestingly, Mares *et al.* (1992) observed a similar lack of effect on the PPF ratio with ketamine, another uncompetitive NMDA receptor antagonist. These experiments therefore further support the

notion that NMDA receptor antagonism (in the absence of HVA Ca^{2+} channel blockade) most likely underlies the anticonvulsant action of DX (this study; Leander *et al.*, 1988; Aram *et al.*, 1989; Chapman and Meldrum, 1989; Roth *et al.*, 1992; Akaike and Himore, 1993; Löscher and Hönack, 1993).

Summary and conclusions

The sigma receptor ligands employed in the present study all demonstrated concentration-dependent anticonvulsant activity against Mg^{2+}-free epileptiform activity in the rat hippocampal slice. The present study documents the novel anticonvulsant actions of both loperamide and opipramol, while the anticonvulsant potencies of the remaining compounds agree well with those determined by other investigators in a variety of different tissues and with a variety of models of epileptiform activity. The anticonvulsant activity of the test compounds was associated with μM concentrations, a finding which casts considerable doubt on the belief widely held in the literature that their anticonvulsant activity results from specific binding at nanomolar concentrations to high-affinity σ receptors.

It was evident that compounds which possess relatively potent NMDA receptor antagonist activity, such as DX and the mixed antagonists ifenprodil, DXM and DTG, demonstrated the most potent anticonvulsant potencies. This was not surprising, due to the prominent role the NMDA receptor-channel complex is known to play in the generation and propagation of epileptiform activity. However, it is also well established that classical uncompetitive NMDA antagonists such as PCP, MK-801, ketamine and DX, which bind to the PCP site of the NMDA receptor with high affinity, induce deleterious neurobehavioural side

effects. More recently identified compounds, including ifenprodil, DXM and DTG, have been found to bind with a lower affinity to the PCP site and, due to their more rapid rates of unblock (Church *et al.*, 1994c), lack the negative side effects seen with the higher-affinity compounds (Rogawski *et al.*, 1991; Porter and Greenamyre, 1995). Accordingly, future anticonvulsant compounds which include NMDA receptor blockade as part of their spectrum of activity should also demonstrate rapid unblocking kinetics so as to minimize deleterious side effects.

It was anticipated that compounds which possess blocking actions at both the NMDA receptor and at HVA Ca^{2+} channels might have demonstrated anticonvulsant IC_{50} values below those required for either NMDA receptor blockade or HVA Ca^{2+} channel antagonism alone. This however was not the case, with the compounds which possess both NMDA and HVA Ca^{2+} channel blockade displaying IC_{50} values between those required for selective NMDA or HVA Ca^{2+} channel blockade. One (perhaps the most likely) explanation for this result lies in the possibility that the spectrum of HVA Ca^{2+} channel blocking activity of the test compounds may in fact be too broad, resulting in blockade not only of excitatory synaptic transmission, but also of inhibitory synaptic transmission. It remains to be established whether a compound which, like adenosine, can selectively inhibit glutamate release, and in addition can antagonize the NMDA receptor and/or postsynaptic VSCCs, would demonstrate maximal anticonvulsant effects at minimal concentrations, resulting in a positive separation of anticonvulsant actions and deleterious side effects. It does seem likely however, that mixed NMDA/Ca^{2+} channel blockers, whose presynaptic Ca^{2+} channel blocking actions target only excitatory neurotransmission but fail to alter inhibitory transmitter release, would be expected to reduce seizure discharges more effectively and with lower concentrations than agents which nonspecifically diminish all transmitter release.

In terms of epilepsy itself, the ability of compounds possessing NMDA receptor antagonist actions or HVA Ca^{2+} channel blocking properties to block epileptiform activity confirms the critical role each channel type plays in the generation and propagation of epileptiform activity. The relative contribution of each system is difficult to assess from this work, although the higher potency of agents which act primarily as NMDA receptor antagonists may suggest that NMDA receptor-channels play a more substantial role in epilepsy than do HVA Ca^{2+} channels.

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