Erosion of inhibition contributes to the progression of low magnesium bursts in rat hippocampal slices

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1. Bathing slices of rat hippocampus in media containing no magnesium ions results in epileptic discharges that originate in hippocampal area CA3. These discharges increase in severity gradually over periods of hours.

2. The progression of epileptic activity was much slower than the equilibration of extracellular magnesium activity and the resulting increase in strength of monosynaptic NMDA receptor-mediated excitation. Its time course matched that of a progressive decrease in pharmacologically isolated, evoked $GABA_A$ receptor-mediated inhibitory postsynaptic current (IPSC) in the CA3 pyramidal cells. Conductance decreased to $37 \pm 6\%$ of control values after 4 h. Responses to exogenous GABA application decreased to $52 \pm 12\%$.

3. Maximal IPSC conductance in 0 mM extracellular Mg^{2+} ($[Mg^{2+}]_o$) also decreased gradually when epileptic activity was abolished by bath application of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D-2-amino-5-phosphonovaleric acid (D-APV) throughout the 4 h incubation period. It reached $61 \pm 8\%$ of control values, a significantly smaller decline than that seen after 4 h of epileptic activity.

4. The decrease in mean IPSC conductance only partially reversed when the recording electrode contained 100 mM Mg^{2+}. Complete recovery of IPSC strength occurred when electrodes also contained either 50 mM MgATP or 20 mM BAPTA. Reintroduction of 1 mM $[Mg^{2+}]_o$ rapidly abolished epileptic activity and caused a slow, partial increase in IPSC conductance.

5. In the presence of 1 mM $[Mg^{2+}]_o$, $GABA_A$ receptor-mediated inhibition had to decrease to $17 \pm 11\%$ of control values, in the presence of 4–7 μM bicuculline, to reach threshold for epileptic activity.

6. These data demonstrate a postsynaptic decrease in $GABA_A$ receptor-mediated inhibition in the *in vitro* low magnesium model of epilepsy. We propose that the apparent leaching of intracellular Mg^{2+} ($[Mg^{2+}]_i$) from cells leads to loss of ATP and consequent partial dephosphorylation of the $GABA_A$ receptor and that this is exacerbated by epileptic activity.

Low levels of magnesium ions in cerebrospinal fluid have been associated with symptoms of clinical epilepsy (Durlach, 1967). More recently, *in vitro* studies have shown that the absence of magnesium ions in extracellular media generates synchronous, epileptic activity in slices of the mammalian central nervous system (Thomson & West, 1986; Walther, Lambert, Jones, Heinemann & Hamon, 1986; Anderson, Lewis, Swartzwelder & Wilson, 1986). It is known that removal of magnesium ions from cerebrospinal fluid dramatically attenuates the voltage-dependent block of the ion channel coupled to the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Removal of this blockade allows an increase in the contribution of NMDA receptors to synaptic transmission (Nowack, Bregestovski, Ascher, Herbert & Prochiantz, 1984; Coan & Collingridge, 1985). This results in strengthening of recurrent excitatory pathways and so generating all-or-none epileptic activity. We have shown

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previously that the enhanced NMDA receptor-mediated excitation alone is sufficient to generate epileptic activity in hippocampal area CA3 (Traub, Jefferys & Whittington, 1994).

In the hippocampal slice preparation it has been shown previously that γ-aminobutyric acid (GABA)-mediated inhibition appeared qualitatively normal during periods of epileptic activity induced by low extracellular Mg^{2+} ($[Mg^{2+}]_o$) (Mody, Lambert & Heinemann, 1987). However, during long periods of exposure to zero $[Mg^{2+}]_o$-containing media, the epileptic activity seen in brain slices increases in severity. In the case of the combined hippocampal–entorhinal slice preparation, occasional polyspike-like events became larger and more frequent, culminating in long periods of spontaneous tonic epileptic activity (Dreier & Heinemann, 1990, 1991). These gradual changes in the form of the epileptic discharge can be mimicked acutely by addition of low doses of the $GABA_A$ receptor antagonist bicuculline to hippocampal slices bathed in low $[Mg^{2+}]_o$-containing media (Traub *et al.* 1994).

The most commonly used experimental epilepsies involve blockade of inhibition. In the neocortex low doses of bicuculline alone are sufficient to generate epileptic activity even with $>80\%$ of GABAergic inhibition intact (Chagnac-Amitai & Connors, 1989). However, the situation in the hippocampus is less clear. Several models of epilepsy are associated with a partial loss of inhibition such as acute application of $GABA_A$ receptor blockers (Schwartzkroin & Prince, 1980), high external K^+ concentration ($[K^+]_o$) (Korn, Giacchino, Chamberlin & Dingledine, 1987; Traub & Dingledine, 1990), tetanus toxin (Jordan & Jefferys, 1992) and kindling (Ben-Ari, Krnjevic & Reinhardt, 1979). Rapid kindling models of epilepsy indicate that run-down of inhibition, due to pathological levels of neuronal activity for long periods of time, is a critical factor in the generation of epileptic activity (Stelzer, Slater & ten Bruggencate, 1987). The mechanism for this long-lasting inhibitory fade appears to involve a change in the phosphorylation state of the $GABA_A$ receptor brought about by an increase in calcium influx through the NMDA-sensitive channel. On a time scale of seconds, decreases in $GABA_A$ receptor-mediated responses accompany activation of NMDA receptors (Stelzer *et al.* 1987) and voltage-operated calcium channels (Inoue, Oomura, Yakushiji & Akaike, 1986). Both these channels play a significant role in the generation of low $[Mg^{2+}]_o$-induced epileptic discharges (Traub *et al.* 1994). It is also known that run-down of responses to exogenously applied GABA also appears to involve MgATP-dependent phosphorylation (Stelzer, Kay & Wong, 1988; Chen, Stelzer, Kay & Wong, 1990; Shirasaki, Aibara & Akaike, 1992). Replacing lost intracellular magnesium ions using intracellular perfusion techniques reverses the time-dependent decrease in GABA response. A decrease in intracellular magnesium ion concentration ($[Mg^{2+}]_i$) has also been reported following traumatic brain injury which itself carries a risk of subsequent epilepsy (Vink, Faden & Mcintosh, 1988)

We hypothesised that low $[Mg^{2+}]_o$ might cause a gradual decrease in GABA-mediated inhibition and that this loss of inhibition could lead to the progressive increase in the severity of epileptic activity. To test this hypothesis we used the *in vitro* hippocampal slice preparation and measured pharmacologically isolated, $GABA_A$ receptor-mediated monosynaptic inhibitory postsynaptic currents (IPSCs) in CA3 pyramidal cells. The effects of low $[Mg^{2+}]_o$-induced epileptic discharges and the effects of changing the intracellular magnesium ion concentration are examined. The importance of these findings with respect to the generation and control of epileptic activity is discussed. A preliminary account of this work has been presented to The Physiological Society (Jefferys, Whittington & Traub, 1994).

METHODS

Forty-one male Sprague–Dawley rats, weighing 250–350 g (Harlan Olac Ltd, Bicester, Oxon, UK), were stunned and killed by cervical dislocation. Transverse dorsal hippocampal slices, 400 μm thick, were cut on a Vibroslice (Campden Instruments, Loughborough, UK), and immediately transferred to a recording chamber at the interface between a warm moist $95\%O_2$–$5\%CO_2$ gas mixture and artificial cerebrospinal fluid (ACSF) flowing at 0·5 ml min^{-1}. The ACSF contained (mM): NaCl, 135; $NaHCO_3$, 16; KCl, 3; $CaCl_2$, 2; NaH_2PO_4, 1·25; $MgCl_2$, 1; D-glucose, 10; and was equilibrated with 95 % O_2–5 % CO_2, pH 7·4 at 34 °C. Epileptic activity was induced by bathing in the above solution without 1 mM $MgCl_2$.

Recording of epileptic activity

Responses were recorded extracellularly using glass micropipettes filled with 2 M NaCl, resistances 5–15 MΩ, from the pyramidal cell layer of area CA3c. Intracellular recordings were taken from seventy-one CA3c pyramidal cells. Conventional recordings were made using glass microelectrodes filled with 4 M potassium acetate or 2 M potassium methylsulphate, resistances 25–55 MΩ. Cells with poor impalements (cell resistance < 30 MΩ, with no action potential overshoot) were rejected.

Monosynaptic $GABA_A$ receptor-mediated IPSCs

Cells were impaled with microelectrodes as above, but filled with an additional 50 mM QX314 (lidocaine *N*-ethyl bromide). Monosynaptic $GABA_A$ receptor-mediated potentials were evoked by stimulating electrodes placed < 50 μm from the recording site in ACSF containing 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20–40 μM), D-2-amino-5-phosphonovaleric acid (D-APV, 50–100 μM) and 3-amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulphonic acid (2-OH-saclofen, 100–200 μM) (Davies, Davies & Collingridge, 1990). All drugs were obtained from Tocris, Bristol, UK. The resulting fast IPSP was clamped using discontinuous single electrode voltage clamp (Axoclamp-2A). The potential dependence of the inhibitory postsynaptic currents (IPSCs) was determined between −100 and −40 mV. From these data the maximum conductance change (g_{IPSC}) and reversal potential were calculated ($n = 5$–7). Data were also recorded with electrodes containing an additional 100 mM magnesium acetate, 50 mM MgATP or 20 mM BAPTA, 30 min after impalement to allow some diffusion of the electrolyte into the cytosol. Resting membrane

potential (−68 ± 1 mV, $n = 87$) and input resistance (42 ± 2 MΩ, $n = 87$) did not change with incubation in 0 mM $[Mg^{2+}]_o$.

Monosynaptic NMDA receptor-mediated EPSPs

Cells were impaled as above, except that the electrode tips were filled with an additional 1 M CsF. EPSPs were isolated by proximal stimulation in the presence of: NBQX (6-nitro-7-sulphamoyl-benzo(*f*)quinoxaline-2,3-dione), 10–20 μM; 2-OH-saclofen, 200 μM; bicuculline, 30 μM; and tetrodotoxin, 2 μM. Cells were injected with hyperpolarizing current to maintain membrane potential at −80 mV to reduce further contamination from calcium channel activation.

Magnesium ion concentrations

The activity of extracellular magnesium ions was measured using single-barrelled, silanized electrodes filled at the tip with magnesium ion-sensitive resin (Fluka ionophore cocktail 63085; Fluka, Gillingham, Kent, UK). Electrodes were calibrated between 0 and 1 mM $[Mg^{2+}]$ before use and gave approximately linear responses across this range (8–14 mV mM^{-1}). The minimum detectable concentration change was 50 μM.

Pressure ejection of GABA

GABA, 1 mM in ACSF, was applied to the slice using pressure pulses (1·2 kg cm^{-2}, 100 ms) from microelectrodes of resistance 0·2–0·5 MΩ. The electrode was placed in the stratum pyramidale and positioned to give a maximal response. Solutions containing GABA at concentrations higher than 1 mM produced longer, but not larger responses, indicating saturation of receptors in the impaled cells.

Data analysis

All responses were digitized (1401; CED Ltd., Cambridge, UK) and stored on computer for off-line analysis (SIGAVG; CED Ltd., Cambridge, UK). All data are expressed as means ± standard error of the mean, or median (interquartile range, IQR). Between five and seven measurements were made for each group. Statistical analyses of changes seen in 0 mM $[Mg^{2+}]_o$ were performed using either Mann–Whitney sign ranked test, Student's non-paired *t* test, or 2-way analysis of variance (ANOVA) followed by Bonferroni's *t* test for multiple comparisons.

RESULTS

Hippocampal slices bathed in artificial cerebrospinal fluid (ACSF) lacking magnesium ions generated spontaneous epileptic discharges after approximately 20 min. Initial epileptic discharges resembled interictal spikes or short polyspikes consisting of an initial primary discharge followed by multiple secondary discharges. During the course of each experiment (4 h) the duration of the primary discharge and the number of after-discharges increased, as did the amplitude and duration of the underlying intracellular depolarization (Fig. 1*A*). Bursts occurring spontaneously after 1 h had a median number of after-discharges of two (IQR, 2–4). There was a significant increase in the number of after-discharges after 3–4 h to seven (IQR, 5–10; $P < 0·05$; Mann–Whitney test; Fig. 1*B*). This progression in severity of epileptic activity was not accompanied by an increase in the size of the NMDA component of synaptic excitation. An increase in monosynaptic NMDA receptor-mediated EPSP amplitude from 2·3 ± 0·3 to 17·6 ± 2·0 mV occurred within 1 h of bathing in 0 mM $[Mg^{2+}]_o$. After 3–4 h the EPSP amplitude was 19·4 ± 0·5 mV. This was not significantly different from measurements taken at 1 h ($P > 0·05$; Fig. 1*B*). The time course of the initial increase in EPSP amplitude appeared to follow the washout of magnesium ions from the extracellular space (Fig. 1*C*). However, the increase in EPSP amplitude reached maximum at around 1 h exposure to medium containing 0 mM $[Mg^{2+}]_o$, whereas the measured extracellular magnesium concentration in the slice had only fallen to 90% of the control level.

The observed progression of the epileptic activity was paralleled by changes in evoked monosynaptic $GABA_A$ receptor-mediated inhibition. A transient increase in maximal IPSC amplitude occurred immediately on washout of $[Mg^{2+}]_o$, lasting approximately 15 min (Fig. 2*A*). This initial transient effect could be abolished by increasing $[Ca^{2+}]_o$ by 1·25 mM to replace the 1 mM $[Mg^{2+}]_o$ (data not shown). This was followed by a slow, sustained run-down of evoked IPSC amplitude, reaching 32% of control amplitudes after 4 h exposure to zero $[Mg^{2+}]_o$ (CNQX and D-APV were present throughout the recording period). This phenomenon was not attributable to the long (4·5 h) duration of the impalement of the cell with the microelectrode because short (20 min) impalements at 1 and 4 h after exposure to 0 mM $[Mg^{2+}]_o$-containing medium also revealed a progressive fade in $GABA_A$ receptor-mediated IPSCs (Fig. 2*B*). This decrease in IPSC amplitude was not attributable to a change in reversal potential for the $GABA_A$ IPSC: control, −68 ± 1 mV; 1 h in 0 mM $[Mg^{2+}]_o$, −69 ± 2 mV; and 4 h in 0 mM $[Mg^{2+}]_o$, −68 ± 1 mV; $P > 0·1$.

Postsynaptic changes in $GABA_A$ receptor-mediated responses

In order to see whether the erosion of inhibition was mediated by pre- or postsynaptic mechanisms we examined the responses of CA3 pyramidal cells to exogenous GABA. Pressure ejection of GABA, optimized to give maximal responses, produced biphasic currents, outward followed by inward, at holding potentials between −60 and −70 mV (Fig. 3). The initial phase of the response, measured at the 80% rise time to minimize contamination with the late component, reversed at slightly more depolarized membrane potentials than the IPSCs: control, −67 ± 2 mV; 4 h, 0 mM $[Mg^{2+}]_o$, −65 ± 1 mV. The late phase had a reversal potential approximately 10 mV more depolarized than the initial response. The estimated conductance associated with the early phase (g_{GABA}) was significantly lower than controls after 4 h incubation with 0 mM $[Mg^{2+}]_o$: control, 122 ± 18 nS; 4 h, 0 mM $[Mg^{2+}]_o$, 63 ± 9 nS; $P < 0·01$ (Fig. 3).

Epileptic activity and the depression of IPSC conductance

To examine the effect of epileptic activity on the run-down of $GABA_A$-mediated IPSCs we compared IPSCs evoked at 1 and 4 h after exposure to magnesium-free media either

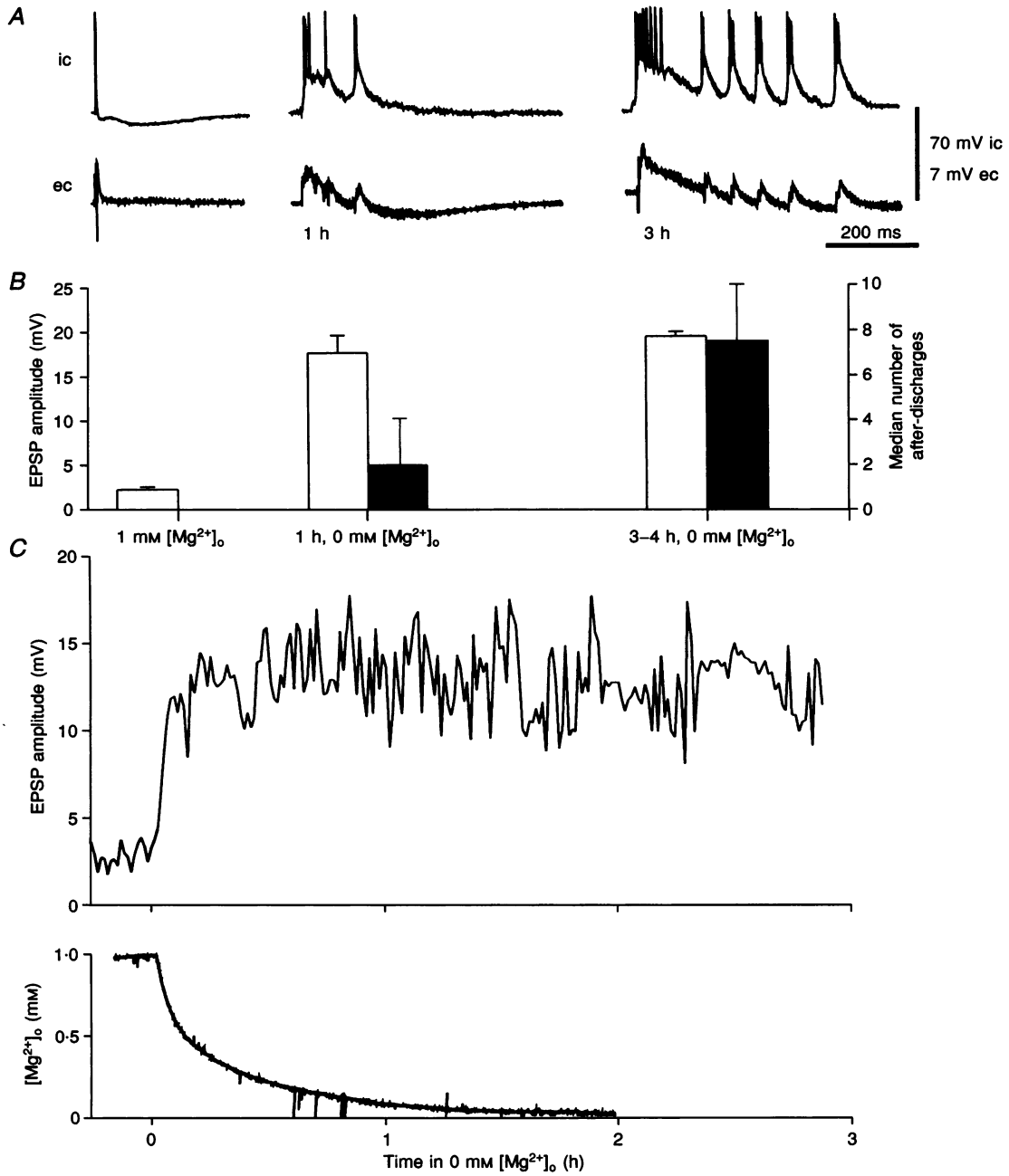

Figure 1. Time course of changes in $[Mg^{2+}]_o$, monosynaptic NMDA receptor-mediated EPSP amplitude and epileptic activity

A, simultaneous intra- and extracellular recordings (ic and ec, respectively) of evoked responses in 1 mM $[Mg^{2+}]_o$ and epileptic activity seen 1 and 3 h after exposure to 0 mM $[Mg^{2+}]_o$. *B*, monosynaptic NMDA receptor-mediated EPSP amplitude (open columns) compared with the median number of after-discharges per burst (black columns). Data are represented as means ± S.E.M. ($n = 5$) and median with interquartile range ($n = 10$). *C*, time course of changes in NMDA receptor-mediated EPSP compared with extracellular magnesium ion activity. Monosynaptic EPSPs were evoked every 45 s using a stimulus intensity sufficient to generate a maximal response in 1 mM $[Mg^{2+}]_o$. Magnesium ion activity was measured at 10 Hz with the electrode placed in area CA3c. Flow rate was 1·5 ml min^{-1} with a tissue bath volume of 0·8 ml. Magnesium activity fell beyond detectable levels within 10 min in the tissue bath alone. A steady, non-biological, DC drift in electrode potential of 1·2 mV h^{-1} was subtracted from the recording.

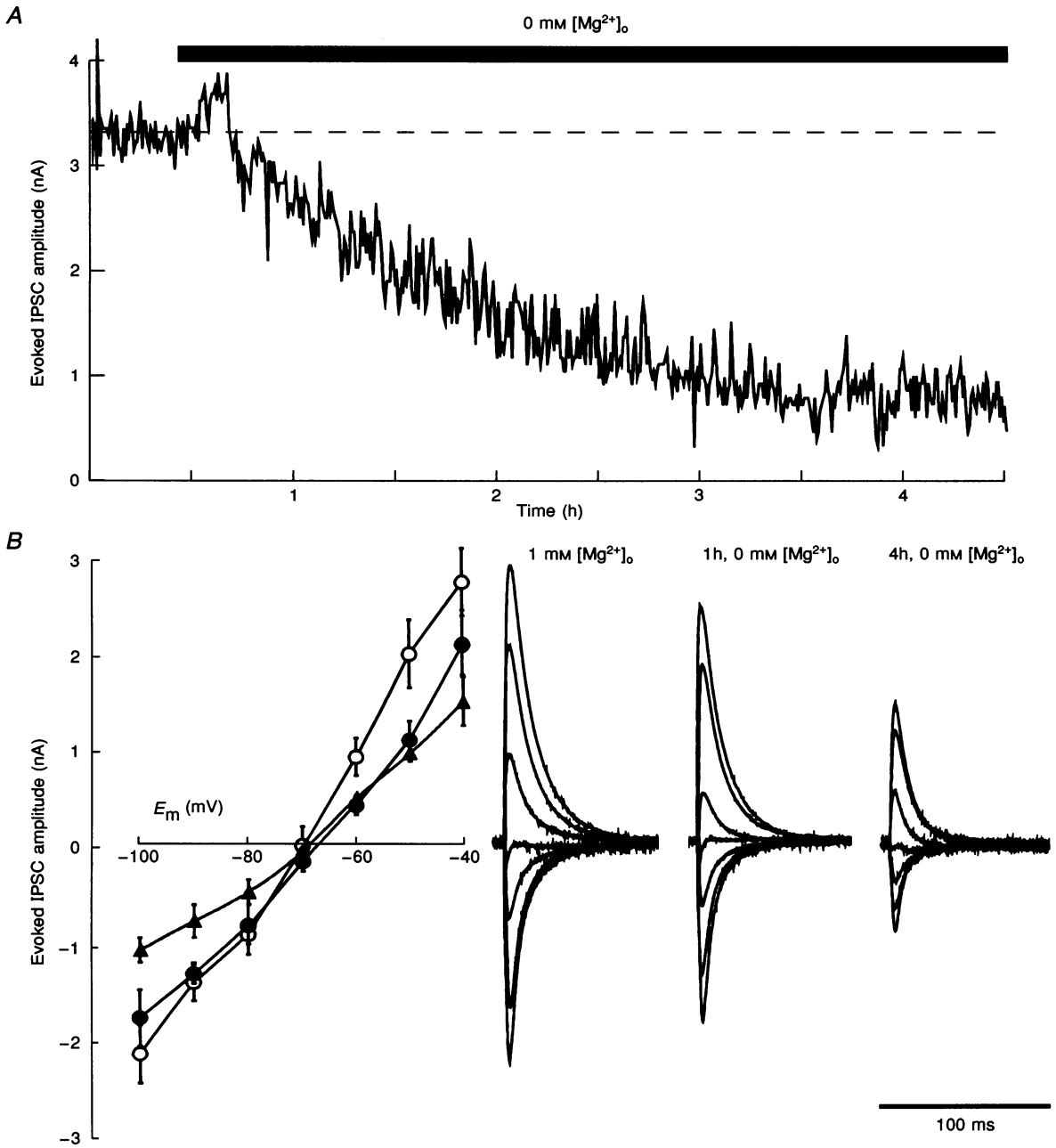

Figure 2. Gradual fade in evoked monosynaptic $GABA_A$ receptor-mediated inhibition concurrent with changes in epileptic discharges

A, single electrode discontinuous voltage clamp recording from a single CA3 pyramidal cell for 4·5 h in artificial cerebrospinal fluid (ACSF) containing CNQX, 20 μM; D-APV, 50 μM; and 2-OH-saclofen, 0·2 mM. Maximal IPSCs evoked from a membrane potential of −40 mV by proximal stimulation every 30 s. Recording electrode resistance 50 MΩ. ACSF was changed to one containing no magnesium ions as indicated by the filled bar. *B*, voltage dependence of maximal IPSCs recorded as in *A*, in 1 mM $[Mg^{2+}]_o$ (○), 1 h (●) and 4 h (▲) after switching to 0 mM $[Mg^{2+}]_o$-containing ACSF. Drugs used to isolate the IPSC were added 10 min before recording. Data shown as means ± S.E.M. ($n = 7$) for each group. Vertical scale for example traces as in vertical axis of the graph.

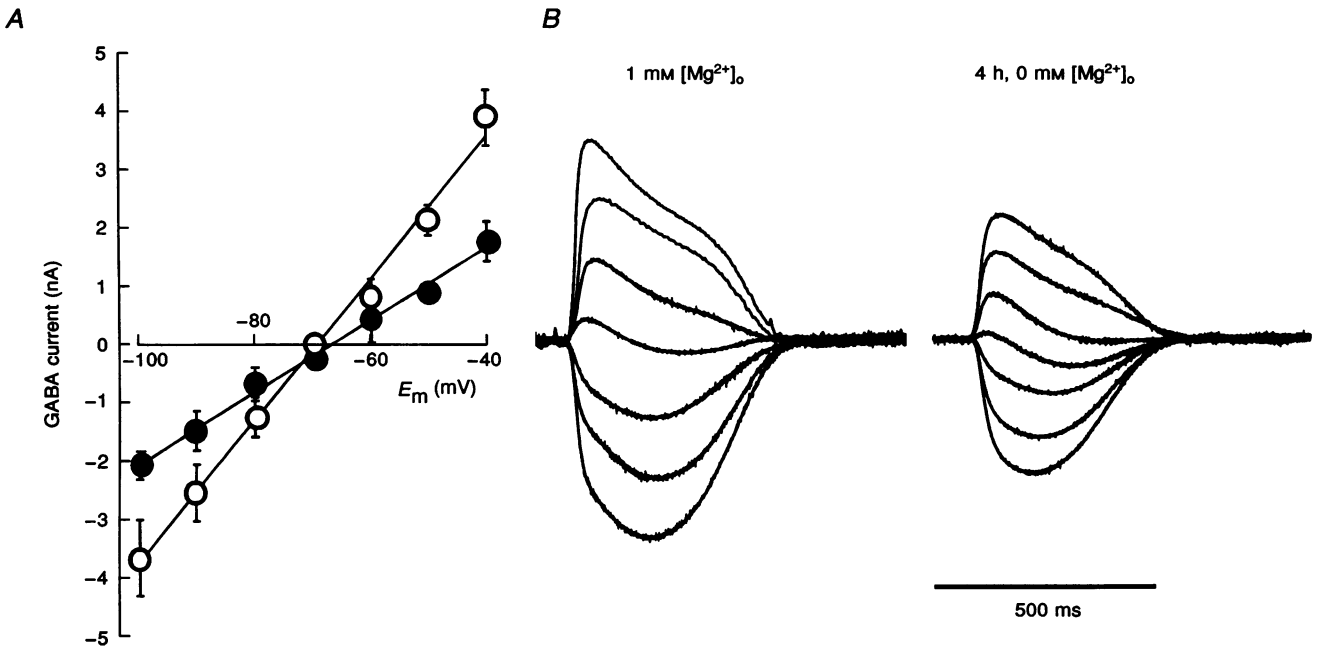

Figure 3. Effects of 0 mM $[Mg^{2+}]_o$ on responses to pressure ejection of GABA

A, current–voltage relationships for the initial phase of the response to 1 mM GABA, applied by a 1·2 kg cm^{-2}, 100 ms pressure pulse. Data shown as means ± S.E.M. ($n = 5$) for responses evoked in ACSF containing 1 mM $[Mg^{2+}]_o$ (○) and after 4 h incubation with 0 mM $[Mg^{2+}]_o$ (●). *B*, families of example traces in the presence (left) and absence (right) of magnesium ions, evoked from −40 to −100 mV, illustrating the biphasic nature of the response and the magnitude of the effects of 0 mM $[Mg^{2+}]_o$. Vertical scale as in the graph in *A*.

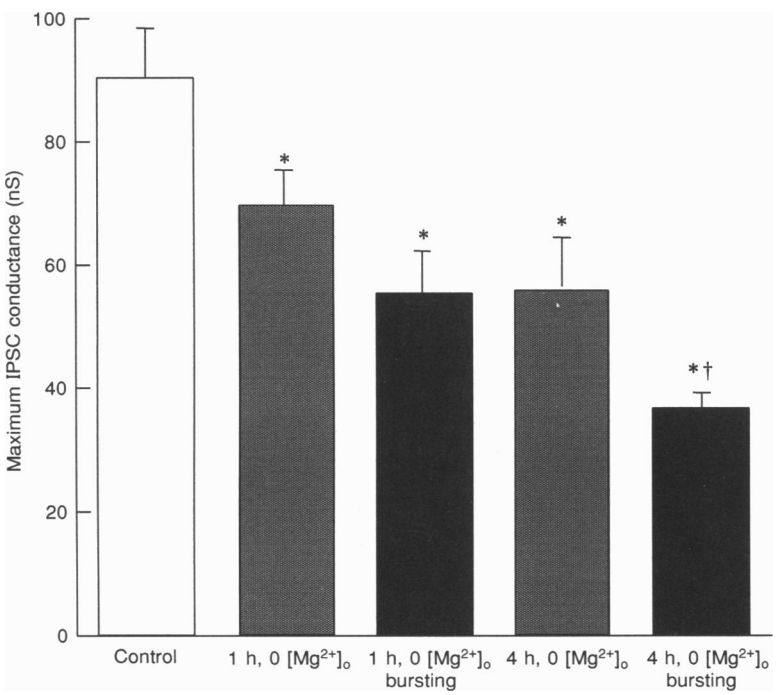

Figure 4. Maximum evoked IPSC conductance in CA3 pyramidal cells: effects of epileptic activity

Data expressed as means ± S.E.M. from: slices bathed in 1 mM $[Mg^{2+}]_o$ (open column, $n = 7$); slices bathed in 0 mM $[Mg^{2+}]_o$ after 1 and 4 h in the continued presence of 20 μM CNQX and 100 μM D-APV (grey columns, $n = 7$ and 5, respectively); and slices bathed in 0 mM $[Mg^{2+}]_o$ after 1 and 4 h of recurrent epileptic activity (black columns, $n = 7$ and 5, respectively). * $P < 0.05$ compared with 1 mM $[Mg^{2+}]_o$ data. † $P < 0.05$, 4 h data with or without previous epileptic activity.

containing no drugs or containing CNQX, D-APV and 2-OH-saclofen to block the epileptic activity (Fig. 4). There was a significant decrease in maximum IPSC conductance after 1 h exposure to magnesium-free medium in both experimental groups. Mean IPSC conductances were: control, 89 ± 8 nS; after 1 h epileptic activity, 54 ± 7 nS; with epileptic activity blocked, 69 ± 5 nS ($P < 0{\cdot}05$ for both groups compared with controls). There was no significant difference between the two 0 mM $[Mg^{2+}]_o$ groups at this time ($P > 0{\cdot}1$). Further decreases in mean IPSC conductance were seen at 4 h with the largest decrease in CA3 cells from slices which had been epileptic for the entire 4 h exposure to 0 mM $[Mg^{2+}]_o$ (IPSC conductance, 36 ± 2 nS). Significantly and substantially less depression of the IPSC conductance occurred between 1 and 4 h when epileptic activity was blocked (IPSC conductance, 55 ± 8 nS; $P < 0{\cdot}05$ compared with data from slices showing epileptic activity for 4 h). The decrease in IPSC conductance, in each case, was not attributable to any change in reversal potential ($P > 0{\cdot}5$, ANOVA with comparisons of all other groups).

Intracellular Mg^{2+} and the depression of IPSC conductance

We suspected that a gradual leaching of magnesium ions from the pyramidal cell cytosol may, in part, have been responsible for the depression of IPSC conductance. IPSCs evoked after 4 h of 0 mM $[Mg^{2+}]_o$-induced epileptic activity were significantly different when recordings were made with microelectrodes containing a number of different solutions ($P < 0{\cdot}05$, ANOVA; Fig. 5*A*). In this series of experiments,

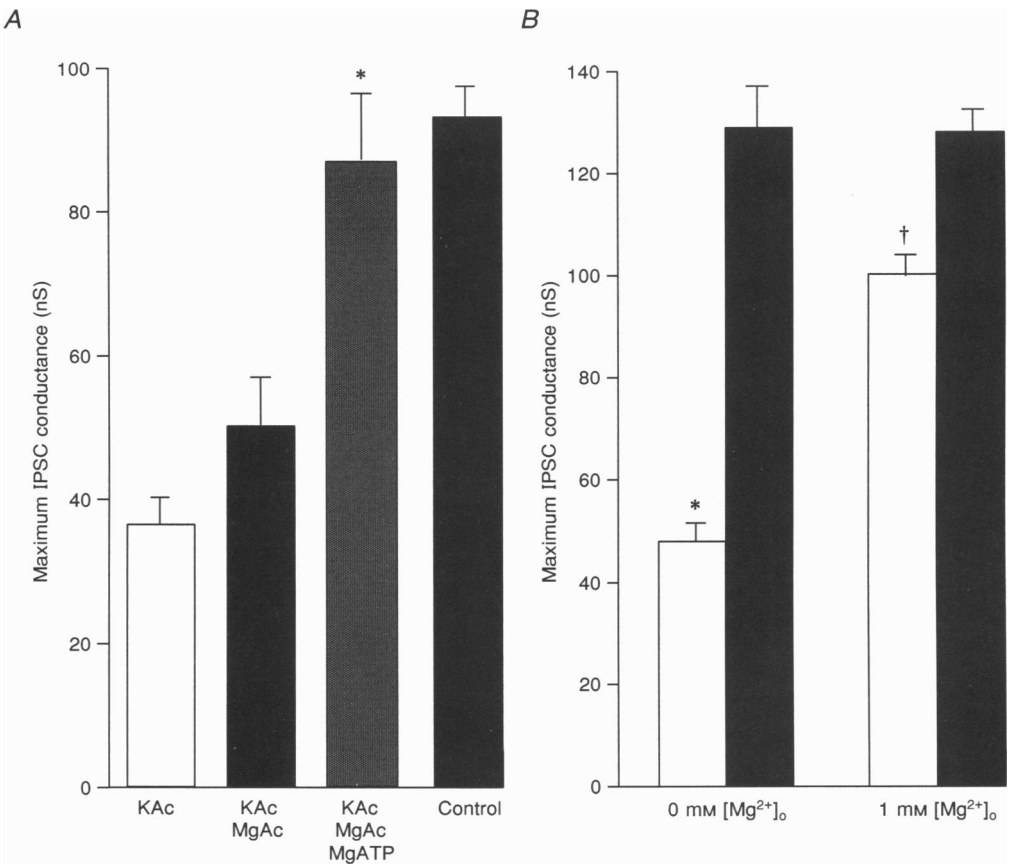

Figure 5. Maximum evoked IPSC conductance in CA3 pyramidal cells: effects of intracellular Mg^{2+}, ATP and BAPTA

A, effects of magnesium ions and ATP on maximal IPSC conductance. Data (means ± S.E.M.) were taken from cells, after 4 h exposure to 0 mM $[Mg^{2+}]_o$, impaled with electrodes filled with 2 M potassium acetate (KAc) (open column, $n = 6$), 1·9 M potassium actetate and 100 mM magnesium actetate (MgAc) (light grey column, $n = 5$), and 1·9 M potassium actetate, 100 mM magnesium acetate and 50 mM magnesium ATP (MgATP) (dark grey column, $n = 5$). Control data (black column) were taken in the presence of 1 mM $[Mg^{2+}]_o$ with electrodes filled with 2 M potassium acetate alone ($n = 7$). $*P < 0{\cdot}05$ compared with conductance measured in 0 mM $[Mg^{2+}]_o$ with electrodes filled only with potassium actetate. *B*, effects of 20 mM BAPTA on maximal IPSC conductance. Data (means ± S.E.M., $n = 5$) were taken from cells after between 3 and 4 h exposure to 0 mM $[Mg^{2+}]_o$ or in the presence of 1 mM $[Mg^{2+}]_o$. Electrodes contained either 2 M potassium methylsulphate alone (open columns) or an additional 20 mM BAPTA (black columns). $*P < 0{\cdot}001$ compared with the effects of BAPTA in 0 mM $[Mg^{2+}]_o$. $\dagger P < 0{\cdot}05$ compared with the effects of BAPTA in 1 mM $[Mg^{2+}]_o$.

using potassium acetate-filled electrodes, IPSC conductance was 35 ± 4 nS (control, 96 ± 8 nS). Addition of 100 mM magnesium acetate to the electrode solution increased the mean IPSC conductance to 49 ± 6 nS after 30 min of impalement, but this effect was not significant ($P > 0.05$, Bonferroni multiple comparison). Further addition of 50 mM MgATP to the electrode solution caused a dramatic increase in IPSC conductance to 85 ± 9 nS after 30 min impalement (Fig. 5*A*). These data were not significantly different from control IPSCs recorded in 1 mM $[Mg^{2+}]_o$ ($P > 0.1$,

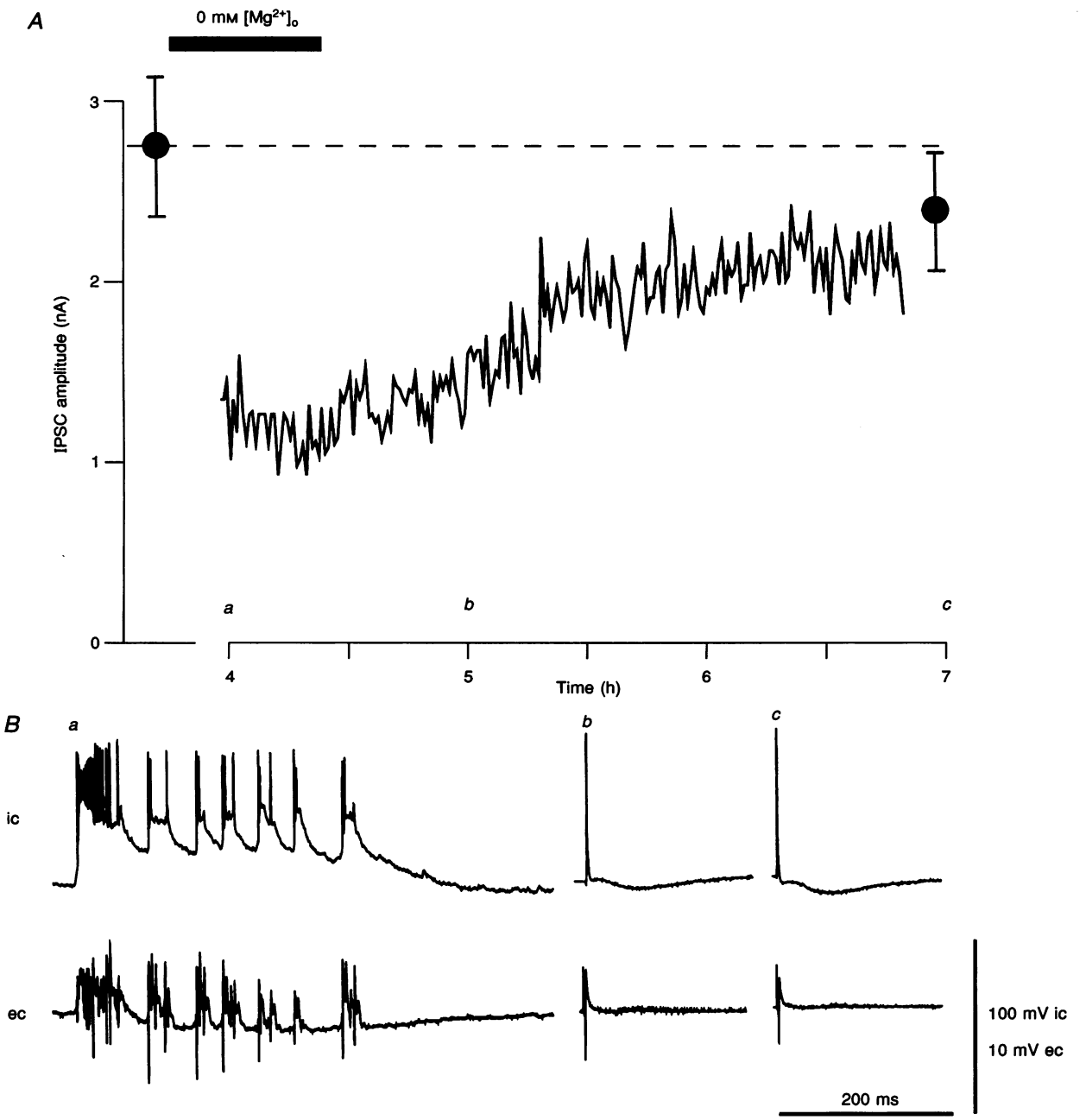

Figure 6. Effects of reintroduction of 1 mM $[Mg^{2+}]_o$ on IPSC and epileptic activity

A, single electrode discontinuous voltage clamp recording from a single CA3 pyramidal cell for 3 h in ACSF containing: 20 μM CNQX; 50 μM D-APV; and 0·2 mM 2-OH-saclofen. Maximal IPSCs evoked from a membrane potential of −40 mV by proximal stimulation every 30 s. Slices were bathed in 0 mM $[Mg^{2+}]_o$-containing ACSF for the previous 4 h of the experiment. 1 mM magnesium chloride was added after 4·5 h incubation in 0 mM $[Mg^{2+}]_o$, at the end of the filled bar above the graph. Initial data indicate means ± S.E.M. of control data ($n = 5$). Dashed line represents mean control IPSC amplitude. Means ± S.E.M. recovery data after 2·5 h in 1 mM $[Mg^{2+}]_o$ are shown on the right ($n = 4$). *B*, simultaneous intra- and extracellular recordings of spontaneous epileptic activity seen after 4 h incubation with 0 mM $[Mg^{2+}]_o$ (*a*), and responses evoked 30 min (*b*) and 2·5 h (*c*) after reintroduction of 1 mM $[Mg^{2+}]_o$.

Bonferroni multiple comparison) indicating that IPSC run-down associated with both low $[Mg^{2+}]_o$ and low $[Mg^{2+}]_o$-induced epileptic activity could be reversed with artificially elevated levels of both magnesium ions *and* ATP (Fig. 5*A*). Again, changes in IPSCs observed were not associated with any shift in the reversal potential ($P > 0{\cdot}05$; Fig. 5*A*).

The effects of calcium-dependent dephosphorylation were examined on IPSCs evoked both in control medium and after 4 h in 0 mM $[Mg^{2+}]_o$. In control medium maximal IPSC conductance recorded in 1 mM $[Mg^{2+}]_o$ was significantly increased from 99 ± 6 to 128 ± 11 nS using electrodes containing 20 mM BAPTA ($P < 0{\cdot}05$; Fig. 5*B*). In 0 mM $[Mg^{2+}]_o$, maximal IPSC conductance was dramatically increased from 47 ± 5 to 129 ± 8 nS ($P < 0{\cdot}001$; Fig. 5*B*). It is interesting to note that in each case BAPTA elevated the maximal conductance measurements to the same level.

The mean IPSC conductance also recovered partially when 1 mM magnesium chloride was added back to the bathing medium after 4·5 h of perfusion with 0 mM $[Mg^{2+}]_o$ (Fig. 6*A*). Conductances increased from $51 \pm 7\%$ of control to $83 \pm 9\%$ after 2·5 h ($P > 0{\cdot}05$). This recovery of the IPSC conductance took considerably longer after application of 1 mM $[Mg^{2+}]_o$ than the cessation of epileptic activity (5–17 min) although the depressed IPSP was still apparent in the compound synaptic potential (Fig. 6*B b*).

Relevance of depression of $GABA_A$ IPSCs to epileptogenesis

In order to put into perspective this profound decrease in $GABA_A$ receptor-mediated inhibition we measured how much disinhibition alone was needed to generate epileptic activity. Slices were bathed in normal ACSF (1 mM $[Mg^{2+}]_o$) containing small doses of bicuculline, increasing in 0·5 μM steps until all-or-none epileptic activity could be evoked (Fig. 7*C*). The threshold dose of bicuculline was between 4 and 7 μM in the presence of 1 mM $[Mg^{2+}]_o$. IPSC conductances associated with these doses of bicuculline

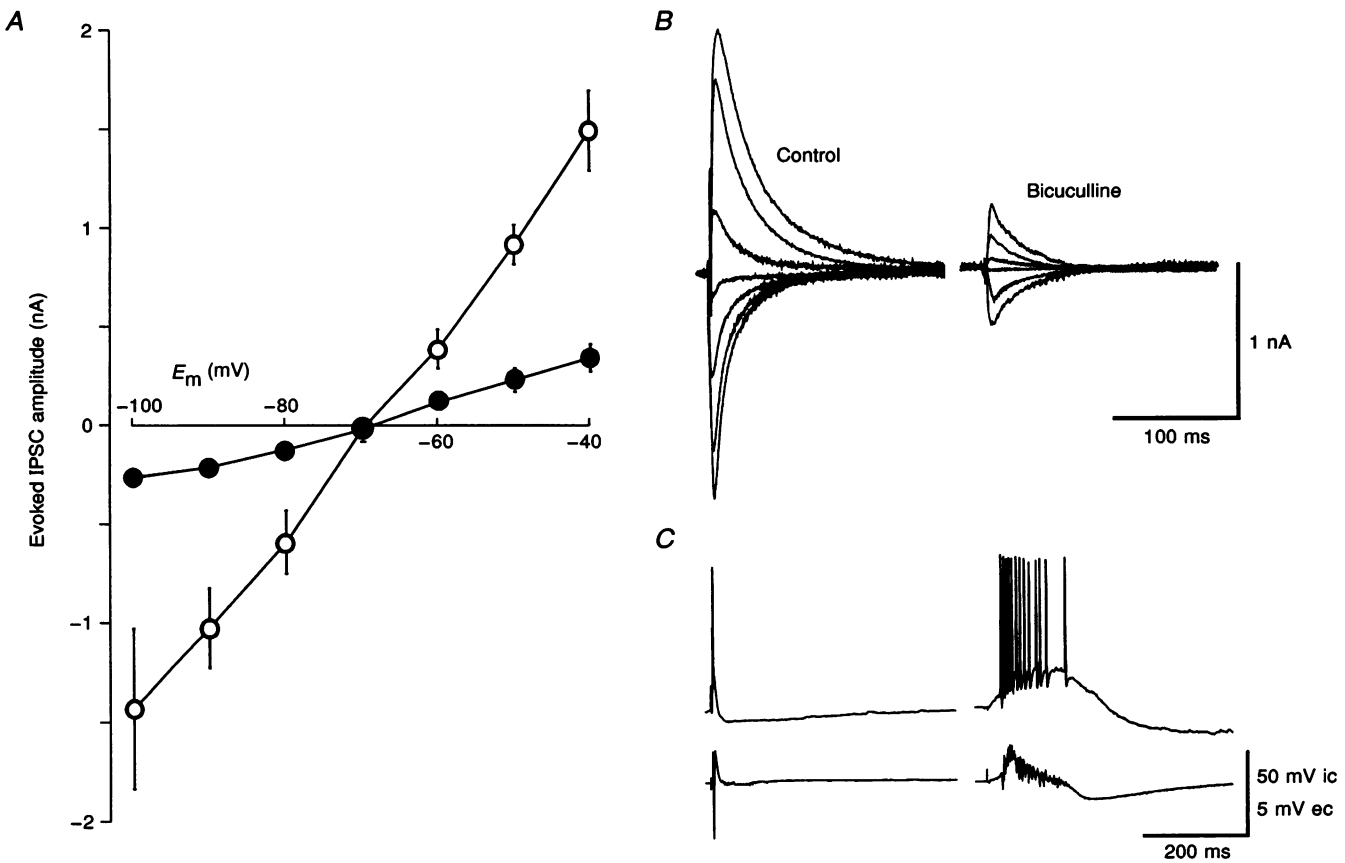

Figure 7. Level of $GABA_A$ receptor-mediated inhibition at threshold for epileptic discharges in 1 mM $[Mg^{2+}]_o$

A, current–voltage plots for IPSCs evoked in CA3 pyramidal cells in ACSF containing 1 mM $[Mg^{2+}]_o$. Bicuculline was added in 0·5 μM doses cumulatively until all-or-none epileptic activity was evoked. Drugs were then added to the bathing medium to isolate the $GABA_A$ IPSC (see Fig. 2). Data (means ± S.E.M., $n = 5$) are shown in the absence of bicuculline (○), and with bicuculline (4–7 μM) at threshold for generating epileptic activity (●). *B*, example traces illustrating the extent of disinhibition required to generate epileptic activity in the presence of magnesium ions. *C*, example of intra- and extracellular recordings of evoked activity without bicuculline and with a threshold dose, in this example 5 μM. Stimulus intensity was 50 V in controls and 10 V in the presence of bicuculline.

decreased from 56 ± 9 to 10 ± 1 nS ($P < 0.05$; Fig. 7*A*). Bicuculline did not affect the IPSC reversal potential. This 83% decrease in evoked monosynaptic IPSC conductance represents a considerable decrease from normal levels of $GABA_A$ receptor-mediated inhibition before disinhibition alone can generate epileptic activity. This degree of disinhibition was greater than that seen after 4 h incubation with 0 mM $[Mg^{2+}]_o$ and explains the rapid restoration of normal excitability on reintroduction of 1 mM $[Mg^{2+}]_o$ without recovery from IPSC erosion.

On-going epileptic activity in 0 mM $[Mg^{2+}]_o$ was considerably more sensitive to disinhibition. In the early (0–1 h) stages of incubation in 0 mM $[Mg^{2+}]_o$, acute application of 1 μM bicuculline was sufficient to mimic the progression of epileptic activity seen at longer incubation periods (Traub *et al.* 1994). Bicuculline at this dose decreased IPSC conductance by 14 ± 4% compared with controls.

DISCUSSION

The present observations demonstrated a gradual erosion of $GABA_A$ receptor-mediated inhibition in CA3 pyramidal cells in the absence of extracellular magnesium ions. This decrease in inhibition caused an increase in severity of spontaneous epileptic activity which, in turn, contributed to further erosion of inhibition. The magnitude of the decrease in inhibition seen after 4 h was not sufficient, in itself, to generate epileptic activity.

The progression of epileptic activity during 3–4 h of exposure to medium containing 0 mM magnesium ions could not be attributed to the time course of removal of the electrostatic blockade of NMDA receptors by magnesium ions. The NMDA receptor-mediated EPSP was maximally potentiated within 1 h, a time when the extracellular magnesium ion concentration had fallen to 10% of control values. The further decrease in $[Mg^{2+}]_o$ from 10% should, from Jahr & Stevens (1990), cause a further increase in NMDA receptor-mediated EPSP amplitude at membrane potentials negative to rest. This was not seen in the present series of experiments. The apparent biphasic nature of the fall in $[Mg^{2+}]_o$ provides one explanation for this discrepancy. The later, slow fall in magnesium concentration may represent the reversal of magnesium transport mechanisms. This would slow the decay of extracellular magnesium concentration but it would also deplete $[Mg^{2+}]_i$ which in turn would decrease the EPSP amplitude due to dephosphorylation of the NMDA receptor (Raymond, Blackstone & Huganir, 1993). Therefore, the stability in monosynaptic NMDA receptor-mediated response could result from a balance between the intra- and extracellular effects of Mg^{2+} on the NMDA receptor.

The observed erosion of $GABA_A$ receptor-mediated inhibition was mainly attributable to postsynaptic mechanisms. Erosion of inhibition occurred with both synaptic activation and pressure ejection of GABA in concentrations sufficient to saturate GABA binding sites. Experiments on dissociated neurones have demonstrated that GABA responses run down rapidly with lowered $[Mg^{2+}]_i$ and $[ATP]_i$ (Stelzer *et al.* 1988), with threshold concentrations of 1·5 mM $[Mg^{2+}]_i$ and 0·5 mM $[ATP]_i$ (Shirasaki *et al.* 1992). $GABA_A$ responses were abolished after only 10 min in the absence of intracellular magnesium and ATP (Chen *et al.* 1990). The above researchers demonstrated the absolute requirement of phosphorylation of a site on the $GABA_A$ receptor on the cytosolic side of the postsynaptic membrane for normal signal transduction to occur. This phosphorylation process is mediated by a kinase, probably protein kinase A (Sessler, Mouradian, Cheng, Yeh, Liu & Waterhouse, 1989; Veruki & Yeh, 1992). The preferred substrate for this enzyme is MgATP, indicating that depletion of $[Mg^{2+}]_i$ would be sufficient to run down $GABA_A$ responses once intracellular stores of MgATP are used up. However, chelation of intracellular calcium with BAPTA restored the IPSC conductance to levels seen with BAPTA in controls, which suggests that phosphorylation of the $GABA_A$ receptor still took place albeit at a lower level. The level of phosphorylation of this receptor is controlled by a dynamic equilibrium between MgATP-dependent phosphorylation and calcium-dependent dephosphorylation (Chen *et al.* 1990).

The much slower, partial run-down of inhibition seen in the present study could be reversed, albeit slowly, by reintroducing 1 mM magnesium into the extracellular medium. A much faster (20 min) recovery of the $GABA_A$ conductance was seen when cells were impaled with electrodes containing magnesium ions and MgATP but not magnesium ions alone (Fig. 5*A*). These observations strongly suggest that the erosion of inhibition seen in the present experiments was generated by a 'leaching' of magnesium ions from cells exposed to magnesium-free media and a consequent decrease in MgATP levels.

The role of disinhibition in the generation of epileptic activity is well characterized *in vitro* (Schwartzkroin & Prince, 1980; Traub, Miles & Jefferys, 1993) and may be involved in the generation of some clinical epilepsies (Lloyd, Bossi, Morselli, Munari, Rougier & Loiseau, 1986). However, the degree of disinhibition seen after incubation with 0 mM $[Mg^{2+}]_o$ was not large enough in itself to generate epileptic activity in the hippocampus. Reintroduction of extracellular magnesium ions rapidly abolished epileptic activity before inhibition recovered. These observations are consistent with increased NMDA receptor-mediated excitation as the essential underlying mechanism of low magnesium-induced bursts (Traub *et al.* 1994).

A decrease in $GABA_A$ conductance of over 80% was necessary to generate epileptic activity in the hippocampal slice. This is in stark contrast to previous observations made in the neocortex which demonstrate an exquisite sensitivity to disinhibition for this tissue (Prince & Connors, 1984; Chagnac-Amitai & Connors, 1989). Here a decrease

in $GABA_A$ receptor-mediated inhibition of less than 20% was sufficient to generate epileptic activity. This difference in sensitivity to disinhibition for the two regions cannot be explained by differing physiological levels of inhibition as neocortical pyramidal cells have $GABA_A$ and $GABA_B$ conductances of similar magnitude to those reported for hippocampal pyramidal cells (Connors, Malenka & Silva, 1988; McCormick, 1989). This suggested either a greater number of, or stronger, recurrent excitatory connections within the neocortex compared with the hippocampus. In the initial stages of the low magnesium model of epilepsy similar small decreases in inhibition (14%) were sufficient to massively potentiate epileptic activity (Traub *et al.* 1994).

Previous studies on pathological levels of neuronal activity revealed a progressive decline of $GABA_A$ receptor-mediated inhibition and have suggested this plays an important role in the generation of epileptic activity (Ben-Ari *et al.* 1979; Stelzer *et al.* 1987). In the present study epileptic activity caused a significantly greater erosion of inhibition than 0 mM $[Mg^{2+}]_o$ with epileptic activity blocked (Fig. 4). A number of possible mechanisms may contribute to this activity-dependent component of run-down of inhibition. Firstly, it appeared from the present data that the slow erosion of inhibition was a consequence of leaking out of magnesium ions, decreasing levels of MgATP in the pyramidal cell and causing a change in the phosphorylation state of the $GABA_A$ receptor. An increase in the energy requirement of a cell, which would occur during epileptic activity, would result in a greater utilization of MgATP and consequently, in the absence of $[Mg^{2+}]_i$, a faster depletion of stores. This change in MgATP levels did not recover with artificial increases in $[Mg^{2+}]_i$ alone, suggesting excessive depletion of high-energy phosphate supplies in these conditions. This is borne out by the experimental data demonstrating that erosion of inhibition could be reversed only by diffusion of MgATP into the cell from the recording electrode. Secondly, epileptic activity involves strong activation of both NMDA receptor-mediated currents and voltage-operated calcium channels (Traub *et al.* 1993). Both these ionophores increase intracellular levels of calcium ions which acutely decrease $GABA_A$ receptor phosphorylation by activation of a calcium- and/or calmodulin-dependent protein phosphatase (Inoue *et al.* 1986; Chen *et al.* 1990). Calcium may also accumulate due to the gradual loss of function of the Mg^{2+}-dependent Ca^{2+}ATPase with decreased levels of $[Mg^{2+}]_i$ (Michaelis, Michaelis, Chang & Kitos, 1983). This increased level of intracellular calcium would be blocked by the calcium chelator BAPTA and, as observed in the present study, would be expected to contribute to the restoration of normal levels of inhibition.

This seizure-dependent erosion of inhibition has important clinical consequences for the understanding of the development of epileptic activity. Decreased $[Mg^{2+}]_i$ and the increased energy requirement of neurones during epilepsy or situations with inherent seizure risk have been reported (Tremblay & Ben-Ari, 1984; Lothman, Hatledid & Zorumski, 1985; Vink *et al.* 1988). From the present findings, erosion of $GABA_A$ receptor-mediated inhibition would be expected to contribute to seizure generation and progression. It is also known that status epilepticus, normally treated with benzodiazepines, becomes more difficult to control the longer the seizures persist (Walton & Treiman, 1988). Such severe epileptic activity would lead to erosion of inhibition by the mechanisms described above. From the present data we can, therefore, suggest that drug resistance after long periods of status epilepticus is a consequence of a dephosphorylation-induced decrease in signal transduction. An increase in the probability of GABA binding in the presence of benzodiazepines (Haefely, 1984) would not be expected to alleviate fully the effects of dephosphorylation of the $GABA_A$ receptor.

The present observations demonstrate that the hippocampus is much less susceptible to disinhibition than the neocortex. They further show that inhibition is labile in the low magnesium model of experimental epilepsy, causing the progression of epileptic activity. More generally this erosion of inhibition provides a mechanism for the evolution of prolonged epileptic activity in clinical conditions.

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