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THE ACTION OF ZINC ON SYNAPTIC TRANSMISSION AND NEURONAL EXCITABILITY IN CULTURES OF MOUSE HIPPOCAMPUS

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SUMMARY

1. The whole-cell configuration of the patch clamp method was used to record from hippocampal neurones in cell culture. Synaptic responses were evoked by loose patch stimulation of adjacent presynaptic neurones in low-density cultures. Agonists and antagonists were applied rapidly, using an array of flow pipes each of diameter 250 μm, positioned within 100 μm of the postsynaptic neurone.

2. Bath application of 50 μM-zinc produced prolonged periods of synaptic barrage and action potential discharge. Flow pipe application of 50 μM-zinc, in glycine-free solution with 1 mM-Mg^{2+}, produced on average a 75% reduction of IPSP amplitude, but increased the average EPSP amplitude to 171% of control. However, after block of γ-aminobutyric acid (GABA) receptors with bicuculline, zinc had no effect on EPSP amplitude, suggesting that potentiation recorded in control solutions reflects block of polysynaptic IPSPs.

3. Consistent with the block of IPSPs postsynaptic responses to flow pipe applications of GABA were blocked by zinc, with fast-on, fast-off kinetics. The equilibrium dissociation constant (K_d) for zinc block of GABA responses, estimated from fit of a single binding site adsorption isotherm, was 11 μM and sufficient to explain the degree of reduction of IPSPs by 50 μM-zinc. Zinc antagonism of responses to GABA was essentially independent of membrane potential over the range -60 to $+60$ mV.

4. With bicuculline methiodide and glycine added to a magnesium-free extracellular solution, to allow the study of synaptic responses mediated by *N*-methyl-D-aspartic acid (NMDA) receptors, zinc reduced the amplitude of EPSPs to 50% of control, and decreased the decay time constant of the EPSP, suggesting that zinc blocks synaptic activation of NMDA receptors.

5. Under conditions where synaptic transmission was completely blocked with postsynaptic receptor antagonists (1–3 mM-kynurenic acid and 10–20 μM-bicuculline methiodide) 50 μM-zinc decreased the amplitude of the spike after-hyperpolarization (AHP), but did not produce large changes in action potential amplitude or half-width. Under these conditions 50 μM-zinc also decreased the current threshold

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required to trigger action potential discharge, and blocked accommodation so that repetitive firing replaced single action potential responses to prolonged current pulses.

INTRODUCTION

A variety of anatomical and chemical techniques show the presence of zinc in the hippocampus of man and subprimate mammalian species (e.g. Frederickson, Klitenic, Manton & Kirkpatrick, 1983). The intense staining of the hippocampal mossy fibre system seen with these techniques (for review see Haug, 1973) seems to have overshadowed earlier studies describing heavy metal staining in many other brain areas. Since ultrastructural analysis shows the presence of zinc in transmitter vesicles (Pérez-Clausell & Danscher, 1985), stimulation of the afferent pathway would be expected to release zinc into the synaptic cleft. Such a release has been reported during depolarization of slice preparations from the hippocampus by electrical stimulation, raised extracellular potassium, or application of the convulsant kainic acid, using radioactive tracer isotopes or atomic absorption spectroscopy as an assay of zinc release (Assaf & Chung, 1984; Howell, Welch & Frederickson, 1984).

In view of the widespread distribution of zinc throughout the brain any action of zinc on neuronal excitability could play an important role in physiological function. However, little is known about the functional role of zinc in the mammalian central nervous system, and only a few experiments have been performed to determine whether zinc has any physiological action on mammalian nerve cells (Smart & Constanti, 1983; Wright, 1984, 1986). In contrast, in experiments on invertebrate axon preparations, the action of zinc on membrane excitability has been studied in considerable detail, and at millimolar concentrations produces block of sodium and delayed rectifier currents (Meves, 1976; Gilly & Armstrong, 1982*a*, *b*). To extend such studies, we examined the action of zinc and the chemically related group IIB cation, cadmium, on synaptic transmission and neuronal excitability in cultures of mouse hippocampus, as well as on responses mediated by agonists acting at excitatory and inhibitory amino acid receptors. The action of zinc on responses to excitatory amino acids is described in the accompanying paper (Mayer, Vyklicky & Westbrook, 1989).

METHODS

Primary dissociated cultures of mouse hippocampal glial cells and neurones were prepared as described previously (Mayer *et al.* 1989). To examine the effects of zinc on spontaneous neuronal activity we used high-density cultures (10^5 cells per dish). To examine stimulus-evoked synaptic responses lower density cultures (12500 or 25000 cells per dish) were used because of the reduced incidence of polysynaptic activity. Cultures of mouse ventral spinal cord were prepared as described by Guthrie, Brenneman & Neale (1987).

Perfusion techniques and solutions

Experiments were performed at room temperature. The recording chamber was perfused at 0·5–2 ml/min, either continuously, or intermittently at intervals of about 30 min. In addition a multibarrel flow pipe was used to perfuse locally the extracellular space around individual

neurones. One barrel always contained the control extracellular solution; drugs were added to the other barrels as required. The flow rate was varied between 30 and 150 μl/min per barrel, depending on the rate of solution change required: fast solution changes for the application of agonists, or for the study of synaptic responses, a more gentle perfusion with a slower exchange rate.

The standard extracellular medium contained (mM): NaCl, 162; KCl, 2·4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; with Phenol Red, 0·01 mg/ml; pH adjusted to 7·3 with NaOH; osmolarity, 325 mosM. In experiments where synaptic responses to the *N*-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptor were studied, $MgCl_2$ was omitted. Various drugs were added as appropriate for individual experiments: 400 nM-tetrodotoxin (TTX) was added in voltage clamp experiments, to block sodium currents; kynurenic acid, 1–3 mM, was used for postsynaptic block of excitatory synaptic transmission; bicuculline methiodide, 5–20 μM, was used for postsynaptic block of γ-aminobutyric acid (GABA)-mediated inhibitory synaptic transmission; glycine, 1–5 μM, combined with 1 μM-strychnine, was used to augment activity of NMDA receptors (Johnson & Ascher, 1987). Zinc chloride, 100 mM dissolved in H_2O with 1 μM-HCl added to dissolve zinc oxychloride, was prepared daily and diluted as required with extracellular medium.

The intracellular solution varied according to the experiment. For the study of synaptic responses, and of neuronal excitability the solution contained (mM): $KMeSO_4$, 140; KCl, 5; HEPES, 10; EGTA, 5; $CaCl_2$, 0·5; $MgCl_2$, 0·5; pH 7·2; osmolarity adjusted to 305–315 mosM with sucrose. To slow inactivation of calcium currents during the study of neuronal excitability, 2 mM-MgATP was added to the intracellular solution. For voltage clamp experiments the intracellular solution was based on CsCl or $CsMeSO_3$. Patch electrodes used for stimulation of presynaptic neurones were filled with $KMeSO_4$ intracellular solution, but with 0·1 mM-Ca^{2+}, no EGTA, and 2·5 mM-Mg^{2+}.

Recording and stimulating techniques

Patch electrodes were used for whole-cell recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981); the electrode resistance varied from 3 to 8 MΩ, depending on the intracellular solution composition. Voltage clamp was achieved with an Axon Instruments 'Axoclamp-2' discontinuous voltage clamp amplifier switching at 8–14 kHz, using gains of 2–4 nA/mV.

When synaptic responses were studied loose patch techniques were used to stimulate presynaptic neurones. Electrodes were first brought in contact with a glial cell to coat the tip with cellular debris and prevent the subsequent formation of giga seals. Subsequently the electrode was brought into contact with nerve cell somata while applying suction to achieve seals of 50–200 MΩ; stimulation was applied using 1 ms current pulses, 10–20 nA, usually with a steady holding current of 0·5–1·5 nA applied to hold the pipette potential at −70 to −80 mV. With this approach, it was possible to stimulate sequentially as many as fourteen neurones in a given microscope field, while recording from one postsynaptic neurone; this greatly aided in raising the probability that synaptic connections could be activated, and once this was achieved, resulted in more stable synaptic responses than could be obtained using whole-cell recording to stimulate the presynaptic neurone.

Results are presented as mean ± standard error of the mean.

RESULTS

Cultures of hippocampal neurones usually fire action potentials spontaneously, reflecting synaptic activity in aggregates of nerve cells; typical responses consist of bursts of synaptic potentials, with evoked action potential discharge, separated by regularly spaced quiescent periods (Fig. 1). Bath application of 50 μM-zinc increased the frequency and duration of bursts of spontaneous activity (Fig. 1), consistent with the convulsant action of zinc applied by intraventricular injection *in vivo* (Donaldson, St-Pierre, Minnich & Barbeau, 1971; Wright, 1986). Similar responses were recorded in three experiments. No obvious change in membrane potential occurred during this excitatory action of zinc, although in other experiments 50 μM-zinc occasionally produced a 2–3 mV hyperpolarization.

The complex nature of such spontaneous activity, which consists of a mixture of excitatory and inhibitory synaptic potentials, together with action potentials and spike after-hyperpolarizations, makes it difficult to determine the site of action of zinc. In order to study the actions of zinc on intrinsic neuronal properties

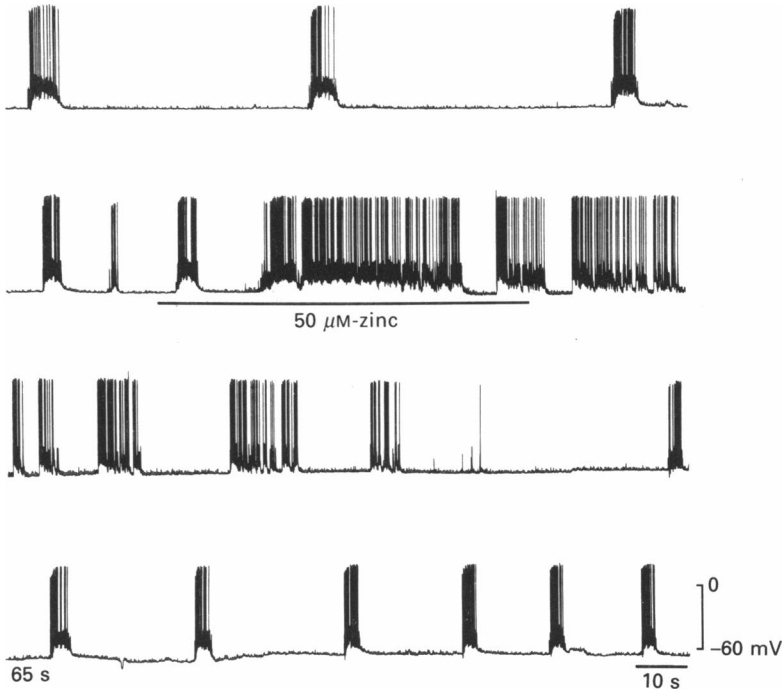

Fig. 1. Zinc increases neuronal excitability. The top three traces show a recording of the membrane potential of a hippocampal neurone during bath application of 50 μM-zinc. The control trace (top) shows three spontaneous bursts of synaptic potentials, with evoked action potential discharge; the middle and lower traces are continuous with the control record, and show prolonged bursting activity evoked by 50 μM-zinc. The fourth trace was recorded 65 s later, and shows the resumption of bursts of spontaneous activity.

intracellular current pulses were used to measure neuronal excitability directly; however, in these experiments action potentials evoked by depolarizing current pulses were usually followed by synaptic responses due to activity in axon collaterals, and their associated disynaptic or polysynaptic circuits. This recruitment of synaptic responses complicated attempts to determine whether the excitatory effect of zinc was due to an action on synaptic transmission, or to a direct effect on the postsynaptic cell, or a combination of these. For this reason subsequent experiments were performed using experimental conditions designed to allow separation of excitatory, inhibitory and intrinsic responses in hippocampal neurones.

Synaptic responses

Stimulation of presynaptic hippocampal neurones via a loose patch electrode frequently evoked short-latency synaptic responses in adjacent cells from which whole-cell recording was obtained. In low-density cultures, polysynaptic activity was greatly reduced, and in many cases the decay of synaptic responses was smooth,

suggesting pure monosynaptic activity (however, see Fig. 3). Both excitatory postsynaptic potentials (EPSPs) and hyperpolarizing inhibitory postsynaptic potentials (IPSPs) were recorded at the resting potential (close to −60 mV), but when IPSPs were studied the membrane potential was normally depolarized to −50 mV with current injection, to increase the amplitude of the synaptic response. In these experiments control responses were always recorded during continuous flow pipe application of extracellular solution, to prevent artifacts due to washing away endogenous modulators of synaptic transmission during application of experimental solutions (e.g. Forsythe, Westbrook & Mayer, 1988).

Flow pipe application of 50 μM-zinc to postsynaptic neurones markedly reduced the amplitude of inhibitory postsynaptic potentials (IPSPs) and at 1 mM-zinc, inhibitory synaptic transmission was completely, though reversibly, blocked. Cadmium, 50 μM, also reversibly blocked the generation of IPSPs, most probably as a result of a block of calcium influx into the nerve terminal (Fig. 2*A*). Similar results to those illustrated were obtained in experiments on five neurones.

Excitatory postsynaptic potentials (fast EPSPs), recorded in glycine-free solution to abolish the slow NMDA-receptor mediated component of the EPSP, were reliably potentiated by application of 50 μM-zinc; with 1 mM-zinc, or 50 μM-cadmium, fast EPSPs were completely, though reversibly, blocked (Fig. 2*B*). Similar results were obtained in eleven neurones.

Stimulation of presynaptic neurones in low-density cultures sometimes evoked obvious polysynaptic responses, consisting of an initial response (e.g. an IPSP), followed by a second response (e.g. a fast EPSP); various combinations were observed. The potentiation of fast EPSPs by 50 μM-zinc could occur via several mechanisms, including a direct action of zinc on excitatory amino acid receptors, or via zinc attenuation of a disynaptic IPSP evoked shortly after the EPSP, and not detected as an inflexion in the EPSP. Clear evidence for the latter was obtained in one experiment, in which an EPSP of latency 7·8 ms was followed by an IPSP of latency 12·2 ms which was selectively blocked during application of zinc (Fig. 3*A*). To confirm that block of IPSPs underlay the potentiation of fast EPSPs by 50 μM-zinc observed in other neurones, we also recorded fast EPSPs in the presence of 10 μM-bicuculline methiodide, to block GABA-mediated IPSPs. When this was done, fast EPSPs were never potentiated by 50 μM-zinc (Fig. 3*B*); similar results were obtained in six experiments.

In cultures of spinal cord and hippocampus bathed in magnesium-free solution, to which glycine has been added, EPSPs are composed of two distinct components (Forsythe & Westbrook, 1988; Forsythe *et al.* 1988), mediated by kainate or quisqualate receptors (fast EPSPs), and NMDA receptors (slow EPSPs). Under conditions which allow expression of NMDA receptor activity, 50 μM-zinc strongly attenuated the amplitude and duration of EPSPs recorded in the presence of bicuculline (Fig. 3*C*); similar results were obtained in five experiments. Since fast EPSPs are not reduced in amplitude by 50 μM-zinc, reduction of the amplitude of these mixed EPSPs must reflect selective attenuation of NMDA receptor activity by zinc (see Peters, Koh & Choi, 1987; Westbrook & Mayer, 1987; Forsythe *et al.* 1988; Mayer *et al.* 1989).

In summary, at low doses zinc (50 μM) strongly attenuates IPSPs (zinc/control =

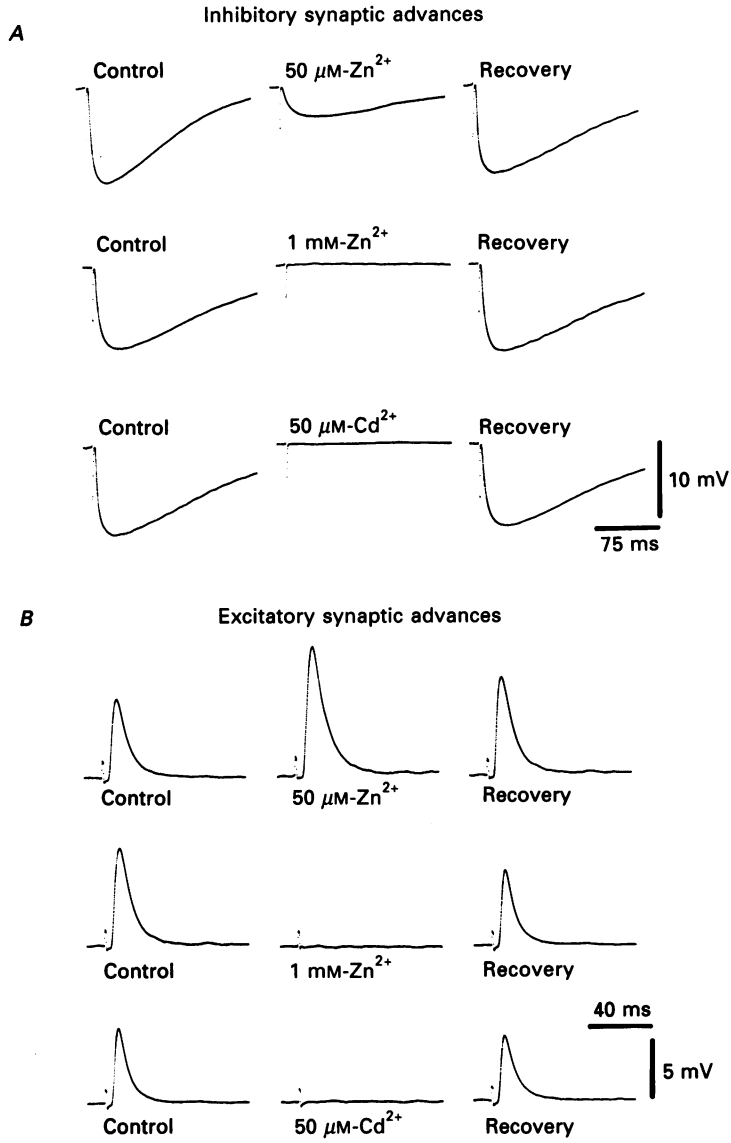

Fig. 2. Zinc blocks monosynaptic IPSPs and potentiates EPSPs. Synaptic responses were evoked by extracellular stimulation of adjacent neurones, using a loose patch electrode. Traces are averaged (twenty to forty responses). *A* shows inhibitory synaptic responses recorded from one hippocampal neurone during sequential applications from a flow pipe of control solution, 50 μM-zinc, 1 mM-zinc and 50 μM-cadmium. Zinc, 50 μM, reduced the mean IPSP amplitude from 12·6 to 3·7 mV, while 1 mM-zinc, and 50 μM-cadmium reversibly blocked inhibitory synaptic transmission. *B* shows excitatory synaptic responses recorded from another hippocampal neurone during sequential applications of control solution, 50 μM-zinc, 1 mM-zinc or 50 μM-cadmium; during the application of 50 μM-zinc the EPSP increased in amplitude, from 6·8 to 11·4 mV, but was reversibly blocked by 1 mM-zinc, and by 50 μM-cadmium. For both *A* and *B* the extracellular medium contained 2 mM-Ca^{2+}, 1 mM-Mg^{2+} and no added glycine, and cells were continuously perfused with control solution, except during application of zinc or cadmium. In *A* the membrane potential was depolarized by DC current injection to −50 mV, to increase the amplitude of the IPSP.

0·25 ± 0·07, $n = 5$), but not fast EPSPs recorded in glycine-free solution in presence of magnesium and bicuculline (zinc/control = 1·06 ± 0·08, $n = 5$). In the absence of bicuculline and glycine, with 1 mM-Mg^{2+}, EPSPs are potentiated by 50 μM-zinc (zinc/control = 1·71 ± 0·16, $n = 11$), reflecting block of disynaptic IPSPs, while EPSPs recorded in the presence of bicuculline and glycine but not magnesium, to enhance NMDA receptor activity, are attenuated (zinc/control = 0·50 ± 0·08, $n = 5$).

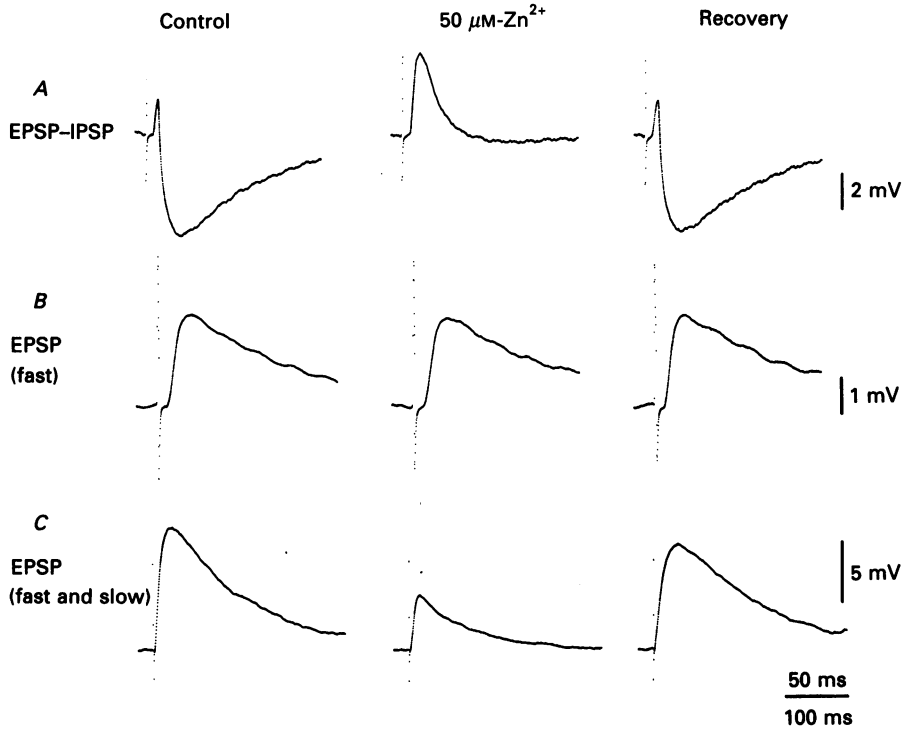

Fig. 3. Pharmacological separation of synaptic responses into zinc-sensitive and zinc-resistant components. *A* shows a mixed EPSP–IPSP sequence, with selective block of the IPSP by 50 μM-zinc, and as a result an increase in amplitude of the fast EPSP. *B* shows a pure fast EPSP recorded in the presence of 10 μM-bicuculline methiodide, with 1 mM-Mg^{2+}, and no added glycine; 50 μM-zinc had no effect on the EPSP amplitude or time course. *C* shows an EPSP recorded in magnesium-free solution, with 15 μM-bicuculline methiodide, and 5 μM-glycine added to the extracellular medium to potentiate NMDA receptor activity; 50 μM-zinc reduced the amplitude and duration of the EPSP. All traces are averaged (twenty to forty responses). Cells were continuously perfused with control solution except during application of zinc.

Zinc and cadmium are GABA antagonists

Block of IPSPs by zinc could reflect either a postsynaptic action of zinc, or a reduction in release of the inhibitory synaptic transmitter, GABA. Zinc and cadmium were found to act as potent antagonists of the response of hippocampal neurones to exogenously applied GABA suggesting that block of IPSPs occurs via a postsynaptic mechanism; a similar block by 50 μM-zinc of responses to GABA but not glycine was found in experiments on spinal cord neurones. The antagonist action of zinc and cadmium showed fast-on, fast-off kinetics, and varied with the

concentration of divalent cation in a concentration-dependent manner consistent with action at a single binding site (Fig. 4). At high concentrations of divalent cation the antagonist action of zinc, and especially cadmium, appeared to saturate at 80–90%, and complete block of responses to GABA was never achieved. In order to

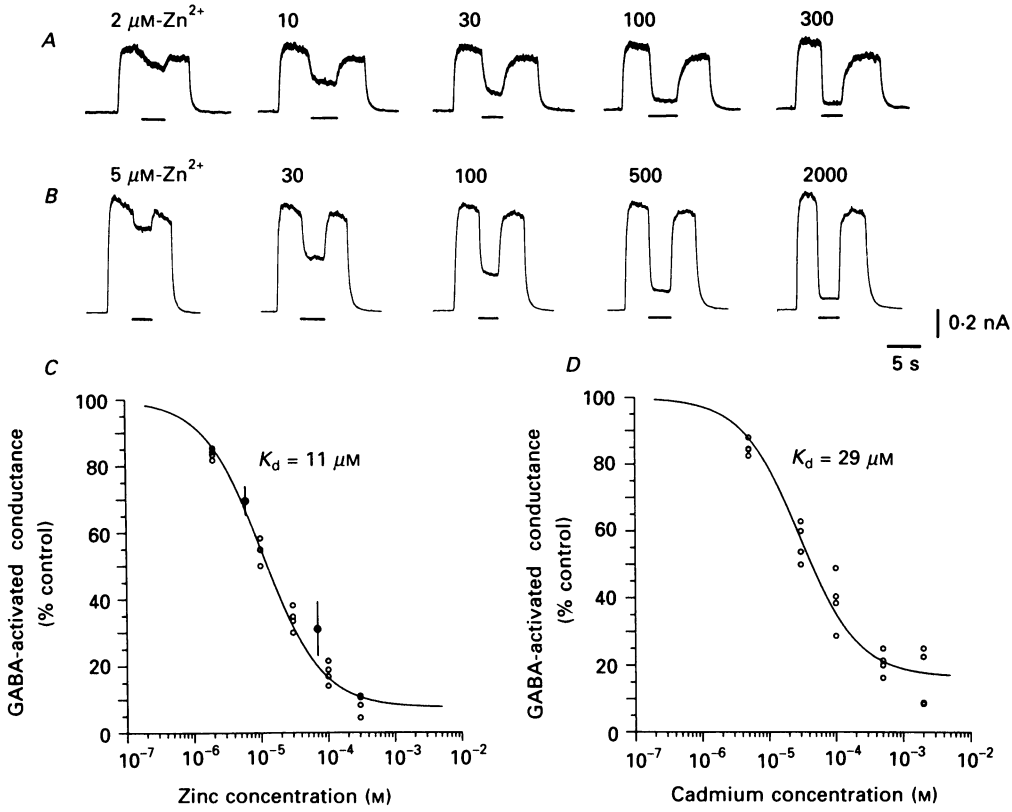

Fig. 4. Zinc and cadmium antagonize responses to GABA. *A* shows dose-dependent antagonism by zinc of responses evoked by application of 3 μM-GABA recorded at a membrane potential of 0 mV, using an intracellular solution containing 140 mM-$CsMeSO_3$. *B* shows a similar, though less potent, dose-dependent action of cadmium. *C* shows data from experiments on four spinal cord neurones fitted with a single binding site adsorption isotherm, assuming a dissociation constant of 11 μM and saturation of zinc antagonism at 92%. ●, responses recorded in experiments on four hippocampal neurones; these values were not included in the dose–response analysis but lie close to the curve obtained for the other points obtained in experiments on spinal cord neurones. *D* shows a similar analysis of cadmium antagonism, assuming saturation at 83%, and a dissociation constant of 29 μM.

estimate the relative potency of zinc and cadmium as GABA antagonists, dose–response curves were obtained and fitted using a modified single binding site adsorption isotherm, of the form

$$I_{\mathrm{GABA\,(Zn)}} = I_{\mathrm{GABA}} \times 1 - \left[\frac{[\mathrm{Zn}^{2+}]}{[\mathrm{Zn}^{2+}]+K_{\mathrm{d}}} \times C\right],$$

where $I_{\mathrm{GABA(Zn)}}$ is the response to GABA recorded in the presence of zinc or cadmium,

I_{GABA} the control response to GABA, K_d the concentration of zinc required for 50% of its maximal effect, and C a constant of maximum value 1 which was used to allow for saturation of the action of zinc or cadmium at less than 100% antagonism. Our experiments show zinc, $K_d = 11$ μM, to be approximately three times as potent as cadmium, $K_d = 29$ μM (Fig. 4).

In turtle retinal cells responses to GABA are blocked by several divalent cations, including cadmium, cobalt and nickel (Kaneko & Tachibana, 1986). These cations all produce a voltage-dependent block of calcium channels (e.g. Lansman, Hess & Tsien, 1986), while cobalt and nickel, but not cadmium, produce voltage-dependent block of the *N*-methyl-D-aspartate subtype of L-glutamate receptor channel in mammalian neurones (Mayer & Westbrook, 1985; Ascher & Nowak, 1988; Mayer *et al.* 1989). However, in the present experiments, block of responses to GABA by both zinc and cadmium showed minimal voltage dependence over the membrane potential range −60 to +60 mV, and could be modelled by assuming that the dissociation constant K_d was only weakly voltage dependent, changing e-fold per 854 mV for zinc, and e-fold per 225 mV for cadmium.

Effects of zinc and cadmium on action potentials

To study changes in action potentials, independent of zinc's action on synaptic transmission, postsynaptic antagonists of excitatory and inhibitory amino acid receptors were included in the extracellular solution. In the presence of 1–3 mM-kynurenic acid and 10–20 μM-bicuculline methiodide neurones were quiescent, EPSPs and IPSPs were completely blocked, and action potentials could be studied in isolation (Fig. 5). Under these conditions the application of 50 μM-zinc from flow pipes never induced action potential discharge, in contrast to results obtained when synaptic transmission was not blocked (Fig. 1). However, measurements with direct electrical stimulation showed an increase in excitability during application of zinc.

There was a small but consistent increase in the action potential amplitude: control, 99·6 ± 1·1 mV; 50 μM-zinc: 102·5 ± 2·3 mV; and in addition a small increase in half-width: control, 1·64 ± 0·04 ms; 50 μM zinc: 1·94 ± 0·04 ms. The spike after-hyperpolarization (AHP) was greatly reduced during perfusion of 50 μM-zinc (Fig. 5*B*), although during the repolarizing phase of an action potential recorded during application of zinc, the membrane potential returned rapidly towards the resting potential compared to the decay of electrotonic potentials (Fig. 5*A*) suggesting little block of the potassium currents underlying the initial phases of action potential repolarization. We were unable to distinguish reliably fast, medium and slow components of the AHP, as has been achieved in experiments on hippocampal brain slices (e.g. Storm, 1987); this might reflect the use of calcium buffers in the intracellular solutions used for whole-cell recording. Compared to control responses the decay time constant of subthreshold depolarizing electrotonic potentials was greatly slowed in the presence of 50 μM-zinc (see Fig. 5*B*), although no effect on the decay of brief hyperpolarizing electrotonic potentials was observed. This suggests that zinc blocks one or more type of potassium current activated just depolarized to the resting potential.

Perfusion of 1 mM-zinc had a more dramatic effect on action potential shape; the action potential amplitude increased slightly, to 103·4 ± 2·6 mV, but the increase in

half-width was more than double the control value (control, $1{\cdot}64 \pm 0{\cdot}04$; zinc $3{\cdot}57 \pm 0{\cdot}26$ ms). The membrane potential depolarization required for action potential initiation was also much larger than in control solution despite a large increase in the input resistance, suggesting an increase in threshold via an action on the sodium current. The spike after-hyperpolarization was completely abolished in the presence

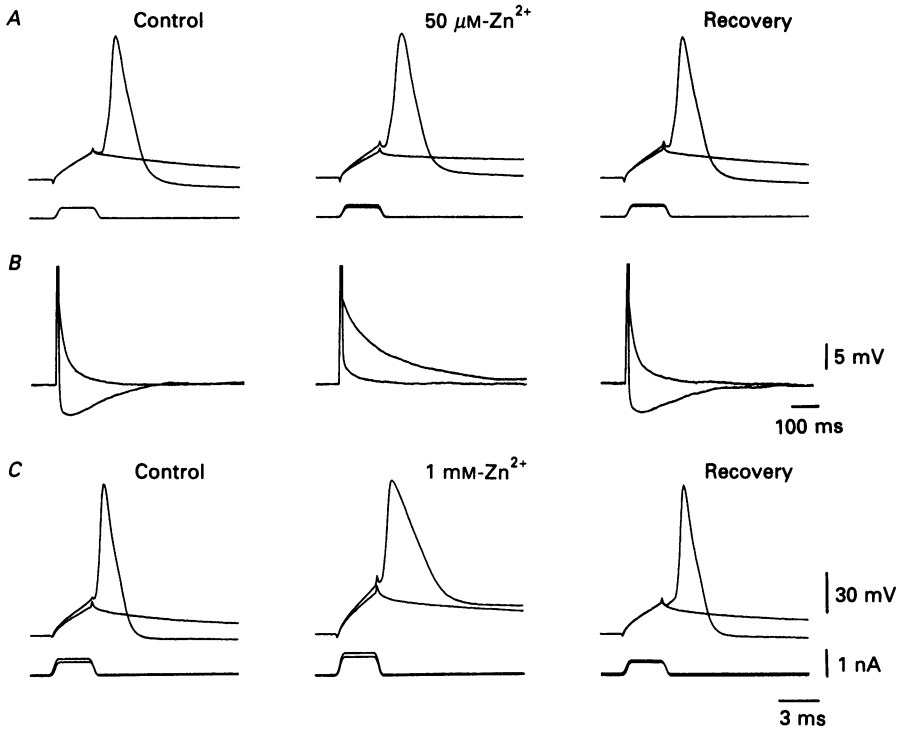

Fig. 5. Zinc modulates action potential characteristics and increases neuronal excitability in the absence of synaptic responses to excitatory and inhibitory amino acids. *A* shows superimposed traces of action potentials and subthreshold depolarizing electronic potentials recorded before, during and after application from a flow pipe of 50 μM-zinc. *B* shows these responses plotted at a slower sweep speed and higher gain to illustrate the effect on the after-hyperpolarization. Application of zinc increased the action potential half-width from 1·85 to 1·9 ms, and greatly reduced the spike after-hyperpolarization. The average value of the decay time constant of subthreshold depolarizing electrotonic potentials increased from 19 ms (control) to 67 ms in the presence of 50 μM-zinc. Note the shoulder on the repolarizing phase of the action potential, and its persistence during application of zinc. *B* shows the increase in action potential width, from 1·4 to 3·0 ms, and the complete block of the AHP, produced by application of 1 mM-zinc. The calibration scales in *C* also apply to the responses shown in *A*.

of 1 mM-zinc, and following the initial fast phase of action potential repolarization the membrane potential decayed slowly towards the resting potential, with a time course similar to the decay of subthreshold depolarizing electrotonic potentials. The effects of 1 mM-zinc were rapidly reversed by perfusion of control solution, and are similar to those expected from previous work on axonal sodium and delayed rectifier potassium currents (Meves, 1976; Gilly & Armstrong, 1982*a*, *b*).

Comparable experiments with cadmium revealed some interesting differences from the effects of zinc. At 50 μM, cadmium also reduced the amplitude of the spike after-

hyperpolarization and produced a small increase in the action potential amplitude, to 101·6 ± 2·6 mV; but in contrast to the effect of 50 μM-zinc, cadmium at the same concentration slightly shortened the action potential half-width from 1·64 ± 0·04 to 1·52 ± 0·10 ms. This effect of cadmium was most obvious as a decrease in the shoulder on the repolarizing phase of the action potential, as if cadmium had blocked an

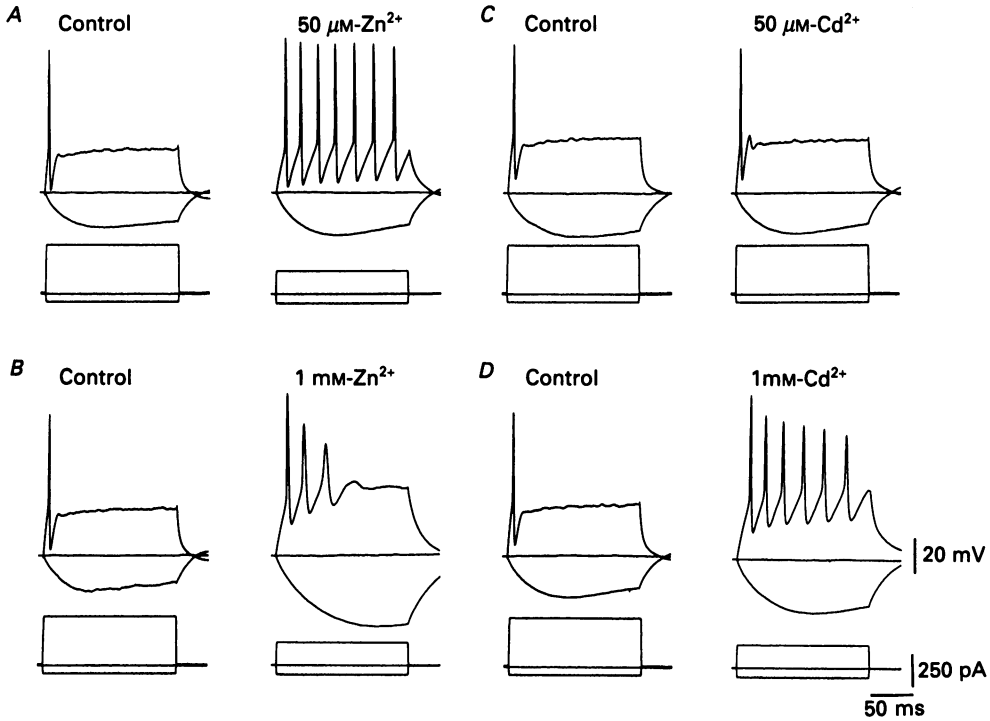

Fig. 6. Zinc increases neuronal excitability in the absence of synaptic responses to excitatory and inhibitory amino acids. The traces show electrotonic potentials and action potentials evoked by current pulses injected into a single hippocampal neurone, bathed in medium containing 3 mM-kynurenic acid and 20 μM-bicuculline methiodide; the patch pipette solution contained 2 mM-MgATP to help maintain calcium currents. *A* shows the effect of 50 μM-zinc. The control trace shows the response to a depolarizing current pulse 1·25 times threshold; repetitive firing was not evoked by further increasing the stimulus strength. During application of 50 μM-zinc the threshold decreased to 0·25 times control, and the neurone fired trains of action potentials in response to a current pulse 1·75 times this new threshold value. In the same cell, similar strength current pulses were used to test for changes in excitability during subsequent perfusion with 1 mM-zinc (*B*), 50 μM-cadmium (*C*), and 1 mM-cadmium (*D*). These effects of zinc and cadmium were rapidly reversible.

inward calcium current; similar results can be seen in some of the records published by Lancaster & Nicoll (1987) and Storm (1987). In contrast, zinc, 50 μM, did not appreciably reduce the shoulder on the repolarizing phase of the action potential (see Fig. 5*A*). At 1 mM, cadmium was similar in action to 1 mM-zinc, and increased spike width at half-amplitude to 2·25 ± 0·23 ms.

Zinc increases excitability by blocking accommodation

One of the most striking effects of low doses of zinc noted after block of synaptic transmission by antagonists was a substantial increase in excitability. In control

solution all neurones displayed marked outward rectification at membrane potentials subthreshold for initiation of action potentials. During perfusion with 50 μM-zinc such rectification was greatly reduced, and as a result the current required to initiate action potentials decreased by a factor of at least 60%. In addition, the majority of hippocampal neurones studied displayed strong accommodation to depolarizing current pulses, and in three of eight neurones this was sufficiently strong to block all repetitive firing after initiation of an action potential, as shown in Fig. 6. In all eight neurones tested the application of 50 μM-zinc strongly reduced such accommodation; this effect was also rapidly reversible. This excitatory action of zinc was strong at 20 μM, and smaller but still readily detectable at 5 μM. In contrast, in two of the three cells in which 50 μM-zinc blocked strong accommodation, and the response to 50 μM-cadmium was also tested, cadmium had no effect at all on accommodation. In these experiments a small membrane potential overshoot did follow the spike after hyperpolarization during perfusion with cadmium, suggesting a weak zinc-like effect (Fig. 6).

High concentrations of zinc and cadmium (1 mM) were much less permissive in increasing excitability. This appeared to result from an increase in the threshold depolarization required for action potential initiation, together with a substantial reduction of the spike after-hyperpolarization; as a result neurones fired two or three rather broad action potentials before becoming refractory. Compared to control responses evoked by depolarizing current injection in the presence of 1 mM-zinc, the membrane potential stabilized at a depolarized value after the cessation of action potential discharge, suggesting that zinc blocks several potassium currents non-specifically. It is likely that the combination of these effects leads to cumulative sodium current inactivation during prolonged depolarizing current pulses, resulting in a progressive decrease in action potential amplitude, action potential broadening, and eventually failure of spike initiation. Cadmium, 1 mM, was intermediate in action between the effects produced by 50 μM- and 1 mM-zinc; excitability increased, and accommodation was strongly reduced, but the spike amplitude decreased during repetitive firing. This is most probably a consequence of cumulative sodium current inactivation resulting from a block of potassium currents, and the associated reduction in the spike after-hyperpolarization. However, a direct action of cadmium on the sodium current is not excluded.

DISCUSSION

Our experiments show that at micromolar concentrations zinc has profound effects on synaptic transmission and neuronal excitability in dissociated cultures of the mouse hippocampus. It is not known whether such effects can occur *in vivo*, as a result of release of zinc from synaptic terminals such as those of the mossy fibres. Important in this regard will be the need to determine whether zinc is released as a divalent cation, or as a complex with carrier molecules; neurochemical experiments demonstrating release of zinc from slices of hippocampus (Assaf & Chung, 1984; Howell *et al.* 1984) did not address this issue, since the assays used determined total zinc release and not the bioavailability of zinc in the extracellular space. In addition, it will be important to determine whether there are differences in the sensitivity of

GABA receptor subtypes in different parts of the brain to the action of zinc, since recent experiments in molecular biology suggest that there exists a large family of GABA receptors. Also, one should consider the possibility that the embryonic and adult forms of GABA receptors differ in their susceptibility to zinc.

Although in cell culture zinc is proconvulsant and blocks IPSPs, spike adaptation, and apparently several potassium currents, the block by zinc of slow EPSPs mediated by activation of NMDA receptors suggests that under some circumstances zinc could also act to decrease excitability in neuronal circuits. Indeed, other NMDA receptor antagonists are potent anticonvulsants (Meldrum, 1986). In addition, the action of zinc on NMDA receptor responses (see Peters *et al.* 1987; Westbrook & Mayer, 1987; Mayer *et al.* 1989), raises the possibility of modulation by zinc of long-term potentiation and synaptic plasticity, at sites where these processes involve activation of NMDA receptors.

Is the potency of zinc sufficient for an action in vivo?

Calculations by Assaf & Chung (1984) suggest that with maximal release the extracellular concentration of zinc could reach 300 μM; the concentrations of zinc used in the present experiments (1 μM to 1 mM) are thus reasonable with what might be expected *in vivo*, under conditions of intense activity. The equilibrium dissociation constant of 11 μM calculated for zinc block of GABA responses would produce 75·4% antagonism of responses to GABA, similar to the average reduction by 75·0 $\pm$ 0·07% of inhibitory postsynaptic potentials produced by 50 μM-zinc. This strongly suggests that zinc block of IPSPs occurs via a postsynaptic mechanism.

The high potency of zinc observed in the present experiments (threshold effects on excitability were seen with 5 μM-zinc) raises the issue as to whether *in vivo*, physiological levels of activity ever produce sufficient release of zinc to achieve concentrations of more than a few micromolar, since in cell culture preparations higher concentrations of zinc are frankly convulsant. However, under patho-physiological conditions the possibility that release of endogenous zinc could contribute to the generation of electrical activity in epileptic foci deserves consideration. Although the first study on the action of zinc as a convulsant stressed its action on Na^+–K^+-ATPase (Donaldson *et al.* 1971), block of inhibitory synaptic transmission and spike accommodation by zinc seem equally likely to be important during application of zinc to the CNS.

Comparison with previous work

Earlier experiments on slices of prepyriform cortex also described excitatory effects of 25–500 μM zinc (Smart & Constanti, 1983), but with several differences from the results described here. In prepyriform cortex EPSPs evoked by stimulation of the lateral olfactory tract were initially prolonged and then irreversibly blocked by bath application of 50 μM or higher concentrations of zinc, while (depolarizing) responses to GABA were potentiated. At the same time that the onset of zinc-induced membrane potential oscillations and enhanced excitability was noted, large spontaneous synaptic potentials occurred, while transmission from the lateral olfactory tract failed. Although the zinc-induced increase in neuronal excitability, which in the olfactory cortex resembles the effect of 4-aminopyridine (Galvan, Grafe

& ten Bruggencate, 1982), appears similar to our results on hippocampal neurones in culture, the irreversible block of excitatory synaptic transmission by 50 μM-zinc, with potentiation of responses to GABA and no effect on the disynaptic GABA-mediated IPSP in the prepyriform cortex, is in contrast to the lack of effect of 50 μM-zinc on EPSPs and the reduction in IPSPs and responses to GABA seen in hippocampal neurones in culture. On the other hand Smart & Constanti (1982) found no action of zinc on responses to GABA recorded in rat sympathetic ganglion neurones, while in the same series of experiments GABA responses evoked in lobster muscle fibres were blocked by zinc. It thus seems possible that $GABA_A$ receptors differ from species to species, and also in different areas of the brain within individual species and perhaps during development. Further experiments are required to resolve this issue, and the species of potassium current blocked by zinc which underlies the reduction of accommodation seen in the present experiments.

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