Differential effect of zinc on the vertebrate GABA_A-receptor complex

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- 1 γ -Aminobutyric acid (GABA) responses were recorded from rat superior cervical ganglia (SCG) in culture using the whole cell recording technique.
- 2 Zinc (50–300 μ M) reversibly antagonized the GABA response in embryonic and young post-natal neurones, while neurones cultured from adult animals were far less sensitive and occasionally resistant to zinc blockade. Cadmium (100–300 μ M) also antagonised the GABA response, while barium (100 μ M–2 mM) was ineffective.
- 3 The differential blocking effect of zinc on cultured neurones of different ages also occurred in intact SCG tissue.
- 4 The GABA log dose-response curve constructed with foetal or adult cultured neurones was reduced in a non-competitive manner by zinc. This inhibition was minimally affected by the membrane potential.
- 5 The GABA response recorded intracellularly from guinea-pig pyriform cortical slices was enhanced by zinc (300–500 μ M), which occurred concurrently with a decrease in the input conductance of the cell. The enhancement was unaffected by prior blockade of the GABA uptake carrier by 1 mM nipecotic acid. This phenomenon could be reproduced by barium (300 μ M) and cadmium (300 μ M).
- 6 We conclude that the vertebrate neuronal GABA_A-receptor becomes less sensitive to zinc with neural (GABA_A-receptor?) development, and the enhanced GABA response recorded in the CNS is a consequence of the reduction in the input conductance and not due to a direct effect on the receptor complex.

Introduction

The γ -aminobutyric acid (GABA) receptor complex in the vertebrate nervous system has recently been established as a multimeric receptor protein with an integral ion channel (Schofield *et al.*, 1988), possessing numerous binding sites for the attachment of benzodiazepines, barbiturates, convulsants and many other modulators of GABA action (Duman *et al.*, 1987; Schwartz, 1988) including divalent cations e.g., zinc (Smart & Constanti, 1982; 1983).

The current interest in zinc as a putative and novel GABA antagonist followed our original observations of a zinc-induced inhibition of the GABA-evoked Cl[−] conductance recorded from crustacean muscle (Smart & Constanti, 1982). The invertebrate muscle GABA receptor was blocked by 10–100 μ M zinc (Smart & Constanti, 1982; Albert *et al.*, 1986). We suggested that zinc was binding to some putative histidine groups resident near the 'mouth' of the GABA-operated ion channel and thereby stabilizing this channel in a closed conformation.

It is quite likely that the invertebrate muscle GABA receptor differs structurally from the vertebrate neuronal GABA_A-receptor, since the latter responds to benzodiazepines and barbiturates and the former does not. In a previous comparison of the action of zinc on vertebrate GABA_A-receptors two unexpected results were observed: firstly, the GABA_A-receptor resident on adult rat sympathetic ganglia was entirely resistant to any blockade by zinc (Smart & Constanti, 1982) and, secondly, GABA responses recorded intracellularly from CNS pyramidal neurones in the pyriform cortex displayed an overt enhancement in the presence of zinc (Smart & Constanti, 1983).

In more recent studies, the action of zinc on GABA_A-receptors would seem to vary with the type of preparation, being either completely ineffective on rat pyriform slices (Hori *et al.*, 1987) and *in vivo* rat cortical neurones (Wright, 1984), or producing a block of GABA responses on frog dorsal root ganglia (DRG) neurones (Yakushiji *et al.*, 1987; Akaike *et al.*, 1987), *in vivo* cat primary afferent fibres

(Curtis & Gynther, 1987) and also on cultured hippocampal neurones of the mouse (Westbrook & Mayer, 1987). These studies of zinc on GABA function suggest that one possible role for the endogenous zinc in the central nervous system (CNS) (Crawford & Connor, 1972; Donaldson *et al.*, 1973) may be to interact with, or modulate the GABA_A-receptor. However, due to the apparent variety of effects zinc is deemed to produce on neuronal GABA responses, we have re-investigated the action of this divalent cation on GABA responses recorded from dissociated and intact sympathetic neurones and brain slices.

Our data suggest that the modulation of GABA responses by zinc critically depends not only on the type of preparation used but also on the stage of neuronal development. Preliminary accounts of this work have been previously communicated (Smart & Constanti, 1988; 1989).

Methods

Sympathetic neuronal tissue culture

Superior cervical ganglia (typically 4–16) were obtained from foetal (E17–21), young post-natal (P1–6) and adult (>P90) Sprague-Dawley rats and dissociated by use of a combination of enzymatic and mechanical methods, as previously described in detail (Smart, 1987). Minor modifications to the protocol included: coating dishes with a 20 μ g ml^{−1} laminin solution (Flow Labs.) (incubated at room temperature for at least 2 h); use of 7S nerve growth factor (from mouse submaxillary glands, 50 ng ml^{−1}, Calbiochem) in the L-15 based growth medium and inhibition of Schwann and fibroblast cell growth was controlled by a 24 h incubation in 10 μ M cytosine arabinoside (Sigma).

Preparation of intact sympathetic ganglia

Intact superior cervical ganglia (SCG) were isolated from urethane anaesthetized (1.5 mg kg^{−1}) Sprague-Dawley rats (200–250 g) (Adams & Brown, 1975). The desheathed ganglion was

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pinned down onto a Sylgard base and perfused with Krebs solution containing (mM): NaCl 118, KCl 4.7, MgCl_2 1.2, CaCl_2 2.5, NaHCO_3 25 and glucose 11; bubbled with 95% $\text{O}_2/5\%$ CO_2 , pH 7.4 at 20–24°C.

Brain slice preparation

Transverse (450 μm) slices of pyriform cortex were prepared from Albino guinea-pigs (250–600 g) with a Campden Vibroslice/M tissue cutter as previously described (Constanti & Sim, 1987) and superfused with oxygenated Krebs solution (23–25°C) containing (mM): NaCl 118, KCl 3, CaCl_2 1.5, NaHCO_3 25, MgCl_2 1.2 and glucose 11; bubbled with 95% $\text{O}_2/5\%$ CO_2 , pH 7.4. Occasionally, slices remained viable even after a 24 h pre-incubation; no obvious differences were detected between recordings made on these slices and those made on freshly dissected slices.

Electrophysiology

Whole-cell recording Patch electrodes (1–5 M Ω) were formed from borosilicate glass and heat-polished to a final diameter of 0.5–1.5 μm . Experiments were performed by the whole-cell recording (wcr) configuration with a List EPC7 patch-clamp amplifier. The cells were continuously superfused in the culture dish with a HEPES-Krebs solution containing (mM): NaCl 140, KCl 4.7, MgCl_2 1.2, CaCl_2 2.5, glucose 11 and HEPES 5; pH 7.4 at 25°C. Phosphate or sulphate salts were omitted to avoid precipitation of the divalent transition metals. The recording pipette solution contained either: (mM) KCl 30, K Asp 120, MgCl_2 1, EGTA 0.5, CaCl_2 0.28 and HEPES 5; buffered to pH 6.8 with 1 M NaOH (final concentration 3 mM); or: KCl 150, MgCl_2 1, EGTA 0.5, CaCl_2 0.28 and HEPES 5; buffered to pH 6.8 with 1 M NaOH. Data were recorded on a Brush-Gould 2400S ink-jet pen recorder. GABA was applied to the neurones either via bath application or by pressure injection from a patch pipette filled with 50 μM GABA, positioned approximately 20–50 μm from the cell soma. Ejection pressures were typically 30–150 kPa for 10–100 ms at 0.1 Hz. Healthy neurones had typical resting poten-

tials in the range of –45 to –75 mV and spike amplitudes > 100 mV. All drugs were obtained from Sigma or BDH Ltd.

Intracellular recording Intracellular recordings were performed on pyramidal cells from olfactory cortical layers II–III (Martinez *et al.*, 1987), or from intact sympathetic ganglia by a Dagan 8100 preamplifier (Constanti & Galvan, 1983). Intracellular electrodes were filled with 4 M potassium acetate (60–80 M Ω). GABA was applied either via bath superfusion or by iontophoresis, using 1 M GABA acidified with 1 M HCl to pH 3–4. The iontophoretic electrode was positioned as close as possible to the intracellular electrode and iontophoretic pulses applied at 1–2 min intervals with positive ejection currents (5–200 nA) and a retaining current of –10 nA. The position of the GABA pipette was adjusted until a 3–15 s GABA application (10–30 nA) evoked consistent, brief depolarizations at the resting membrane potential (typically –80 mV for olfactory neurones). Higher ejection currents for more prolonged periods were avoided to minimize any shifts in the GABA equilibrium potential or overt GABA receptor desensitization (Kriegstein & Connors, 1986). Data were recorded on a Brush-Gould 2400 chart recorder. Healthy cells used in this study had resting potentials of –75 to –85 mV and spike amplitudes > 90 mV for CNS neurones, and –45 to –55 mV resting potential and spike amplitudes > 70 mV for ganglionic neurones.

Results

Zinc inhibits GABA-induced membrane currents on foetal and young post-natal cultured superior cervical ganglia neurones

The bath application of GABA (1–20 μM) to foetal rat cultured SCG neurones routinely induced an inward membrane current and an increase in the membrane conductance recorded under whole-cell voltage clamp (Figure 1a). The inward current was also associated with an increase in membrane current noise (Figure 1a). Generally, the GABA response was

Figure 1 Effect of zinc on GABA-evoked membrane currents recorded from embryonic (E21) cultured superior cervical ganglia (SCG) neurones under whole-cell voltage clamp. (a) Bath-applied GABA induced an inward current (I_{GABA} ; upper trace) at a holding potential of –55 mV with an associated conductance increase observed following voltage commands to –75 mV (300 ms, 0.1 Hz; lower trace). An increased membrane current noise was also evident. (b) Zinc (200 μM) was applied for 10 min and induced a small outward current and dramatically inhibited I_{GABA} which was reversible after a 6 min wash (w 6 min) (c). All drugs were applied for the duration of the solid lines in this and subsequent figures. The chart speed was increased every 10 s for the duration of each voltage command pulse.

After recovery from initial bath-applied GABA doses, the cell input conductance was frequently reduced (a), but usually after only one dose of GABA it attained a steady state and was not further reduced following recovery from subsequent GABA applications (c).

well maintained during the perfusion of GABA (1–2 min), exhibiting only a slight propensity for fade or desensitization. Following the washout of GABA, the input conductance of the cell was typically depressed and occasionally would never recover to the pre-GABA level (Figure 1a). GABA responses after bath application were quite reproducible exhibiting only a slow 'run-down' with time after formation of the whole-cell recording mode (cf. Stelzer *et al.*, 1988; Figure 1a and c). The application of zinc (100–300 μM) to cultured sympathetic neurones produced a small outward current and a small reduction in the input conductance. Additionally, the inward GABA current and conductance increase were also markedly reduced (Figure 1b). In 100 μM zinc, GABA responses were inhibited by approximately $57 \pm 1.7\%$ (mean $\pm$ s.e.mean, $n = 25$ cells, cultured from either E21 or P1–5 age rats). The GABA response quickly recovered on zinc

washout (Figure 1c). Zinc was always effective in blocking the GABA responses recorded from all the foetal and young post-natal (P + 1–5) cultured SCG neurones used in this study ($n = 30$).

GABA was also routinely applied via pressure ejection from a patch pipette juxtaposed to within 50 μm of a whole-cell voltage-clamped neurone. This mode of drug application allowed many more GABA responses to be recorded from a single cell with virtually no 'run-down' over 2–3 h recording. Reproducible GABA-induced inward currents (evoked at 0.1 Hz) peaked rapidly in less than 100 ms, concomitant with an increased membrane current noise, and decayed to the control resting current level usually within 1–3 s. In many cells, higher frequency emission of GABA (up to 4 Hz) did not promote any obvious reduction in the peak of the GABA responses (desensitization) which eventually summated to

Figure 2 Effect of group IIa and IIb divalent cations on the GABA-induced inward current (I_{GABA}) in embryonic cultured superior cervical ganglia (SCG) neurones under whole-cell recording mode at -55 mV holding potential. GABA was pressure applied (75 kPa, 30 ms, 0.1 Hz) from an adjacent patch pipette 50–100 μm from the cell. Group IIb cations, zinc (Zn, a) and cadmium (Cd, b) were equi-effective and reversible GABA antagonists, whereas barium (Ba), a group IIa cation, was ineffective (c). The chart recorder was slowed during the recoveries from zinc and cadmium. Divalent cations were applied for approximately 2 min. (d) Zinc did not enhance GABA desensitization on SCG neurones. Desensitization of the GABA response was induced by repetitive pressure ejections of GABA (77.2 kPa, 50 ms, 0.2 Hz) onto an SCG neurone voltage clamped at -55 mV. Sequential GABA responses in control solution were slowly reduced with time (d (i)); in the presence of 50 μM zinc (d (ii)), this slow decay was unaffected, as observed by plotting the logarithm of GABA current amplitudes against time from the start of the pressure applications (e). In (e), (○) control; (●) + 50 μM zinc.

form a smooth current. Figure 2a illustrates that the antagonism of GABA responses by zinc was not limited to just bath-applied GABA, since responses to GABA applied by brief pressure were also readily antagonized by zinc (100–300 μM) causing a reversible reduction of 51% in this cell.

To test whether the inhibition of GABA responses was a unique feature of zinc, we also studied the effect of cadmium, another transition divalent metal ion also belonging to group IIb. This ion, at similar concentrations, was just as effective as zinc in promoting a reversible inhibition of the GABA-evoked membrane current (Figure 2b; 55% inhibition; $n = 3$). However, this inhibitory action was not a general feature of all divalent cations since the group IIa divalent ion, barium, did not inhibit the GABA response over a wide concentration range (100 μM –2 mM; $n = 3$) (Figure 2c).

GABA responses recorded from cultured sympathetic neurones showed only a slight propensity to desensitize or fade (cf. Figure 1a). Whether zinc was inhibiting the GABA response by enhancing desensitization was studied by measuring the decay rate of GABA responses evoked by repetitive pressure applications of GABA. The decay rate in normal Krebs was a slow process (112.5 ± 6.7 s; $n = 3$; Figure 2d(i)) and may monitor only part of the desensitizing or fading phenomenon. It was unlikely to reflect a shift in the GABA reversal potential, since the conductance change also declined simultaneously (cf. Figure 1a). Zinc 50 μM produced a reduction in the evoked GABA currents (Figure 2d), but clearly did not affect the decay time constant (122.7 ± 5.5 s; $n = 3$; Figure 2e).

This inhibition of GABA responses by zinc also occurred on foetal rat sensory ganglia cultured under identical conditions. Dissociated cultures of rat dorsal root ganglia (from the thoracic and lumbar spinal regions; age E21) yielded two main cell populations based on cell diameter. GABA responses were readily observed following pressure ejection, although the sensitivity to GABA (measured as the amount of inward current and conductance recorded under voltage clamp) seemed considerably lower on these sensory ganglionic neurones compared to the sympathetic cultures of an equivalent age. Bath application of 50 or 100 μM zinc inhibited the GABA responses by $29.3 \pm 6.7\%$ and $60.6 \pm 5.2\%$ ($n = 5$ cells) respectively on both the major neuronal cell types.

The reduction of GABA responses by zinc on sympathetic neurones was most probably due to a direct effect on the GABA receptor/channel and not apparently due to a shift in the GABA reversal potential (E_{GABA}), which remained unaltered (Figure 3a and b). Also, the GABA chord conductance was inhibited by zinc in an apparently voltage-independent manner (Figure 3c). The lack of any fade in the GABA response recorded in many neurones also indicated that the increased transmembrane chloride ion flux induced by GABA (Adams & Brown, 1975) probably did not cause any substantial shift in E_{GABA} under wcr conditions (where usually $[\text{Cl}^-]_0 = [\text{Cl}^-]_i = 152$ mM providing an $E_{\text{GABA}} = 0$ mV; for potassium aspartate intracellular solutions, $[\text{Cl}^-]_i = 32.5$ mM yielding an $E_{\text{GABA}} = -40$ mV).

Currently, the blockade of GABA responses by zinc has been observed mostly in cell cultures or enzymatically-treated cells (Akaike *et al.*, 1987; Westbrook & Mayer, 1987). Whether this block could be a phenomenon generated solely in dissociated cell culture, or by enzyme treatment (Inoue & Akaike, 1987) was investigated by utilizing young manually desheathed post-natal intact rat SCG (P + 5–6). The potency of zinc as a GABA antagonist was now assessed on a preparation possessing an integral cytoarchitecture including both pre- and postganglionic nerve trunks.

Intracellular recording from these ganglia and ionophoretic application of GABA revealed a monophasic depolarization and conductance increase which was clearly dose-dependent and readily reversible. The application of 100 μM zinc caused a small inhibition at high GABA ionophoretic doses, but on increasing the zinc concentration to 300 μM , a more overt non-competitive inhibition of the GABA dose-conductance rela-

Figure 3 Current-voltage (I-V) relationships for an embryonic cultured superior cervical ganglion (SCG) neurone voltage clamped at -55 mV. (a) Control I-V relation was constructed in the absence ($\square$) and presence ($\blacksquare$) of 5 μM GABA. The GABA-evoked current, at different command potentials, was obtained by subtraction of the two curves and plotted as the chord conductance in (c). (b) In the same cell following recovery from the initial dose of GABA, two further I-V relationships were measured in the presence of 100 μM zinc ($\circ$) and also 100 μM zinc + 5 μM GABA ($\bullet$). (c) (i) The GABA chord conductance in the absence ($\circ$) and presence ($\bullet$) of 100 μM zinc was plotted against voltage. The GABA reversal potential was -37 mV. The effect of zinc was essentially devoid of any voltage sensitivity over this potential range, reducing the GABA chord conductance by 48% at -55 mV and 40% at -135 mV membrane potential (ii) shows a plot of the % reduction in GABA chord conductance against membrane potential. Note the reduction in control GABA conductance at hyperpolarized potentials.

tion was apparent, similar to that observed in cultured neurones (*vide infra*). Previously, in intact adult rat SCG, we showed that bath-applied GABA responses were completely unaffected by zinc (100–500 μM ; Smart & Constantini, 1982).

The present results with foetal cultures and the young intact SCG, prompted us to reinvestigate the action of zinc on intact adult rat ganglia and also on adult SCG cultures obtained from animals of a similar age ($> P + 90$).

Zinc is less effective as a GABA antagonist in adult sympathetic neurones

Bath application of $50 \mu\text{M}$ GABA to an adult SCG neurone (2–3 months old), recorded under current clamp, resulted in a membrane depolarization and conductance increase (Figure 4a). Larger doses of GABA were now required to observe any response, due to the presence of avid GABA uptake mechanisms in this tissue (Bowery *et al.*, 1979). Zinc (200 – $500 \mu\text{M}$) slightly hyperpolarized the cell, similar to the effects observed on foetal cultured neurones, and also increased the resting input resistance. However, GABA responses were unaffected by zinc, as previously found (Smart & Constanti, 1982) (Figure 4b and c). The apparent lack of effect of zinc was not due to poor penetration into the desheathed ganglion, since spontaneous transmitter release increased in zinc as observed by the appearance of 'membrane potential noise' (Figure 5c). Also, zinc induced the appearance of anode break spikes following the cessation of the hyperpolarizing electrotonic potential in the presence of GABA (Figure 5c; cf. Smart & Constanti, 1982).

The intact mature preparations were clearly less sensitive to zinc than either the intact young post-natal SCG or the cultures of foetal neurones. This reduced sensitivity of GABA responses to zinc blockade also applied to mature neurones cultured from 'equivalent-age' adult animals (age $> P + 90$).

Pressure-ejection of GABA onto cultured adult neurones, produced a rapid inward current followed by a 'noisy' decay back to the holding current analogous to the GABA response recorded in foetal neurones. By using similar pressure ejection parameters for GABA to those used on foetal neurones, bath application of zinc now produced a much smaller reduction in the GABA response (Figure 5b) (32.5% reduction in this cell). In contrast, foetal SCG neurones cultured for a similar period of time (15 DIV; Figure 5a(i)) to the adult neurones (20 DIV; Figure 5b) exhibited GABA responses that were more substantially blocked by identical concentrations of zinc (67.5%) (Figure 5a).

The reduced sensitivity of adult neuronal GABA responses to zinc was occasionally manifest as a complete resistance to zinc blockade ($n = 5$ cells) (Figure 6), comparable to our observations on the intact adult SCG. Figure 6a shows GABA

responses obtained on adult cultured neurones following pressure ejection of GABA. Zinc ($100 \mu\text{M}$) did not inhibit the GABA response even when the concentration was increased to $300 \mu\text{M}$ (Figure 6b), which in foetal SCG neurones will elicit an 80% reduction in the GABA response. However, $300 \mu\text{M}$ zinc did induce a small increase in neuronal excitability, signalled by the presence of > 1 spike associated with each GABA current (arrow, Figure 6b). The input conductance of the neurone was only slightly decreased by $300 \mu\text{M}$ zinc, which may have been the cause of a small apparent enhancement in the GABA response ($16.8 \pm 2.1\%$) observed occasionally and only when the GABA response was not blocked by zinc (Figure 6b) ($n = 5$ cells). Interestingly, barium ($500 \mu\text{M}$ – 2 mM) caused a larger reduction in the input conductance of these adult cells and the GABA responses were also frequently enhanced by 5–10% (data not shown). These small and infrequent enhancements of GABA action by zinc or barium on cultured ganglion neurones were routinely observed more clearly on intact cortical brain slice GABA responses and may have a similar underlying mechanism (see Figure 11).

The apparently 'zinc resistant', adult ganglionic GABA receptor was a *bona-fide* member of the GABA_A-receptor subtype family, since GABA responses were readily blocked entirely and reversibly by $10 \mu\text{M}$ (+)-bicuculline methochloride (Figure 6c). These data suggest that the GABA receptor in the rat SCG may undergo subtle developmental changes affecting function, which can partly be resolved by the action of zinc on the GABA-induced Cl^- current. This postulated developmental modification was highlighted further by comparing dose-response curves obtained for GABA on adult and foetal cultured SCG neurones in the presence and absence of zinc.

Foetal cultured SCG neurones were exposed to pressure ejected GABA and a dose pressure-response curve was constructed by varying the ejection pressure. The GABA log dose-response curve was depressed by $100 \mu\text{M}$ zinc in a non-competitive manner, exhibiting a small increase in the degree of blockade at higher agonist doses (Figure 7a). At low agonist doses the percentage reduction in the GABA response was 50% and this increased to 65% at the top of the curve.

In contrast, on adult cultured SCG neurones, a plot of the peak GABA-induced conductance change against log GABA concentration revealed that zinc was now considerably less effective, although a non-competitive mode of action was still appropriate (Figure 7b). The inhibitory effect of zinc also showed a slight dependence on agonist concentration, reducing the GABA response by 27% at low GABA doses ($10 \mu\text{M}$) and by 33% at higher concentrations ($40 \mu\text{M}$).

Figure 4 The GABA response is insensitive to zinc in intact adult rat superior cervical ganglia (SCG). (a) Intracellular recording of current clamp responses to bath applied GABA which consistently evoked a small depolarization (upward deflection) and conductance increase (reduced hyperpolarizing electrotonic potential amplitude evoked by -0.16 nA , 300 ms , 0.2 Hz current pulses). (b) Bath application of $200 \mu\text{M}$ zinc caused a small hyperpolarization and reduction in membrane conductance, but no inhibition of the GABA response after 15–25 min. (c) Increasing the zinc concentration to $500 \mu\text{M}$ still did not produce any GABA-inhibition, but caused a further small hyperpolarization and the appearance of some spontaneous action potentials together with an increased baseline 'noise'.

Figure 5 GABA responses are differentially sensitive to inhibition by zinc in rat cultured superior cervical ganglia (SCG) neurones obtained from foetal or adult animals. GABA was applied by pressure application (76 kPa; 50 ms, 1 pulse 30 s^{-1}) to embryonic cultured neurones (a(i)) at -55 mV under whole-cell voltage clamp. Bath application of zinc induced a rapid and reversible inhibition of the inward GABA-evoked membrane currents, shown on an expanded time scale in (a(ii)). By performing a similar protocol with neurones cultured from adult rats (P + 90) (b) and with similar ejection pressures (63 kPa, 30 ms, 1 pulse 30 s^{-1}) for GABA, it was revealed that zinc was much less potent as a GABA antagonist.

Figure 6 Zinc is completely ineffective as a GABA antagonist in some adult rat cultured superior cervical ganglia (SCG) neurones. (a, b) Whole-cell voltage clamp records of the GABA-induced inward current (I_{GABA}) recorded following pressure ejection of $50\text{ }\mu\text{M}$ GABA (75.2 kPa, 50 ms, 1 pulse 30 s^{-1}). The downward deflection beyond the inward current represents an uncontrolled, truncated sodium current (arrow) due to the cell firing an action potential in response to the GABA-induced current. Membrane conductance was monitored by applying hyperpolarizing voltage commands from -55 mV holding potential (-10 mV , 300 ms, 0.03 Hz). GABA responses were completely unaffected by bath application of $100\text{--}300\text{ }\mu\text{M}$ zinc which eventually caused the appearance of multiple uncontrolled spikes (b(i) arrow). (b(ii)) Shows selected records at higher time resolution. In the same cell after washout of zinc, I_{GABA} was completely abolished by $10\text{ }\mu\text{M}$ (+)-bicuculline (Bic) (c(i)), but it recovered on washout after 8 min; shown on expanded time scale in (c(ii)).

Figure 7 GABA dose-response curves recorded from foetal (a) and adult (b) rat cultured superior cervical ganglia (SCG) neurones by whole-cell voltage clamp. GABA was applied by pressure ejection, varying either the pressure or pulse duration to achieve a dose-response relationship, and the resulting GABA-induced inward current (I_{GABA}) measured. In control Krebs, the GABA log dose-response curves were sigmoidal. Bath application of 100 μ M zinc resulted in a non-competitive depression in both curves but clearly zinc was more effective on the foetal cells (a). Insets show the percentage reduction in the control GABA response by zinc. The points in (a) are mean values and vertical lines s.e. mean determined from 5 successive exposures to the same pressure application of GABA. In (b) mean values were taken from 3–8 separate determinations. The GABA response was calculated as the mean peak inward current, from at least three successive responses, following the attainment of a steady-state response each time after alteration of either the ejection pressure or pulse duration. The ejection pressures and pulse durations were chosen randomly to avoid any possible cumulative effects of GABA caused by merely incrementing the effective GABA concentration. In (a) and (b), (○) control responses; (●) responses in the presence of zinc 100 μ M.

The insensitivity of the adult ganglionic GABA receptor to zinc implies that other adult GABA_A-receptors, present in CNS tissue, should also be unaffected by zinc. However, our previous study (Smart & Constanti, 1983) revealed an enhancement of GABA responses in the presence of this cation. Does this dual action of zinc suggest the existence of more than one subtype of GABA_A-receptor?

Zinc enhances the GABA response recorded from adult brain slices

Olfactory cortical neurones responded to bath-applications of GABA (1–4 mM; 1–2 min) with a slow depolarization and associated conductance increase (Brown & Scholfield, 1979). In general agreement with our earlier findings (Smart & Constanti, 1983), these responses were clearly enhanced (but never blocked) after a 10–20 min pre-incubation with 300–500 μ M zinc. In addition, zinc also caused a 5–10 mV membrane depolarization associated with a dramatic decrease (up to 50%) in the resting input conductance and an increased incidence of spontaneous neuronal firing, which persisted during the GABA response.

The enhancement of GABA responses by zinc was probably not mediated by an inhibition of GABA uptake carriers (Brown *et al.* 1980), since the effect was clearly observed even in the presence of 1 mM nipecotic acid, a GABA uptake inhibitor (Figure 8) (Johnston *et al.*, 1976). In the combined presence

of zinc and nipecotic acid, GABA now evoked distinctly larger depolarizations and apparent conductance increases relative to control, accompanied by characteristic repetitive neuronal firing. Recovery from the effects of zinc was typically incomplete, despite prolonged washing (up to 1 h).

To determine the time course of the zinc-induced enhancement, GABA was focally-applied to single neurones by microiontophoresis. GABA produced dose-dependent depolarizations at the resting membrane potential (–80 mV), accompanied by large increases in input conductance ($n = 9$ cells). The peak of the GABA response was attained within 5–10 s of iontophoresis, and recovery was complete within a few seconds of terminating the current pulse. When the membrane potential was depolarized to –70 mV, GABA elicited a biphasic response, consisting of an early, small hyperpolarization followed by a larger depolarizing component (Figure 9). A clear increase in the amplitude of the late GABA depolarization and peak conductance change relative to control was observed after 15–30 min in zinc (Figure 9). In addition, some reduction of the early hyperpolarizing GABA response was also apparent, but this may have been masked by the enhanced, and now much larger, depolarizing component. Zinc also induced a progressive fall (47%) in the resting input conductance which was commensurate with the GABA response enhancement (Figure 9d). Concurrently, the cell also began to fire characteristic, spontaneous action potentials. The shape of these spikes was similar to the shape of anti-

Figure 8 Current clamp recording from an olfactory cortical neurone (resting potential -75 mV) showing the enhancement of GABA responses by zinc in the presence of a GABA uptake inhibitor. Hyperpolarizing current pulses (-0.38 nA, 200 ms) were applied throughout at 0.33 Hz and upward deflections are spontaneous action potentials (truncated). (a) A depolarizing response to bath-applied GABA obtained in control solution and after 30 min in nipecotic acid (Nip). (b and c) After 50 min, zinc and nipecotic acid were applied in combination. GABA, applied after 15 or 45 min, now elicited notably larger responses. Little recovery of the GABA response was obtained after a 20 min washout. Open arrows show where the membrane potential was readjusted back to -75 mV.

dromic action potentials evoked in this preparation (Scholfield, 1978), suggesting that they may be generated at a site distal to the soma (uninfluenced by GABA) and retrogradely conducted back towards the somatic recording site. All these effects of zinc were typically slow to reverse on return to normal Krebs solution, requiring >1 h washing for recovery.

Whether this enhancing action of zinc was due to a large shift in the GABA reversal potential was investigated by applying the same ionophoretic dose of GABA at various membrane holding potentials, in the absence or presence of zinc (Figure 10a). The control GABA response, which was depolarizing at -80 mV, became biphasic at -70 mV, whilst at -50 mV, both early and late components were clearly hyperpolarizing. A plot of the GABA response amplitude against membrane potential (Figure 10b) revealed the reversal potential of the early hyperpolarization to be -76 mV, while the later depolarizing component reversed at -60 mV. After 20 min in $300 \mu\text{M}$ zinc, there was little change in the reversal potential of the early hyperpolarization. However, the reversal level of the late component was shifted by $+10$ mV. At potentials negative to -60 mV, the GABA-induced input conductance increases were not being exaggerated by an outward rectification of the resting membrane. Indeed, shifting the membrane potential from -80 to -60 mV (either in control or in zinc solution) revealed only a small (15%) but typical fall in cell input conductance. Interestingly, a sharp increase in the resting conductance, observed between -60 and -50 mV, was apparently inhibited by zinc, suggesting that a voltage-dependent conductance mechanism operating over this voltage range had been blocked.

The temporal correlation between the zinc-induced reduction in the input conductance and the enhancement of the GABA response prompted us to test whether these effects of zinc could be reproduced by other divalent cations, e.g. barium and cadmium. Barium ($300 \mu\text{M}$; $n = 3$) depolarized the membrane by 25 mV and increased the resting input resistance by 160% (cf. Constanti & Galvan, 1983; Figure 11). After

readjustment of the membrane potential to -70 mV, the re-application of GABA now produced enhanced depolarizations and peak changes in input conductance. This apparent enhancement was maintained for as long as barium was present, and was only slowly reversible after a prolonged washout. Some increase in spontaneous (antidromic ?) firing was also observed in barium (Figure 11a), particularly during the washout period, but not so intense as that seen with zinc. There was also a 5–10 mV positive shift in the apparent reversal level of the late depolarizing GABA component (cf. effect of zinc) and the time course of the GABA enhancement was commensurate with the reduction in resting input conductance (Figure 11b). This suggested that the apparent ability of zinc and barium to enhance the GABA response may result from the overall change in neuronal electrical properties following the reduction of the resting membrane conductance.

In contrast, cadmium ($300 \mu\text{M}$; $n = 3$) showed a dual effect on the cortical GABA response, causing an initial (<5 min) enhancement with a small fall in input conductance. However, after 20 min the responses to subsequent GABA applications were consistently reduced in amplitude. Unfortunately, the interpretation of this result was clouded by the development of an irreversible increase in the input conductance in the presence of cadmium.

Discussion

The original reason for this study was, initially, to reaffirm our observation that zinc enhanced cortical GABA responses while not affecting sympathetic ganglionic GABA responses and to attempt to answer the question of why GABA_A receptors located within different regions of the vertebrate nervous system respond differentially to zinc. We believe the answer may be two fold, relying firstly on a possible differentiation of the GABA_A-receptor protein with neuronal development, and secondly, on the spatial distribution of GABA receptors on neurones with vast dendritic networks, particularly in the CNS.

Figure 9 Biphasic responses to ionophoretically-applied GABA (90 nA, 10 s) recorded from an olfactory cortical neurone (resting potential maintained at -70 mV throughout, by direct current injection). Brief (80 ms) hyperpolarizing current pulses (-0.47 nA) were applied at 1 Hz. The control GABA response was very reproducible (a) and after 3, 8, 15 or 32 min superfusion with zinc exhibited a progressive enhancement. An increase in input resistance and spontaneous cell firing were also induced by zinc. (c) Partial recovery of membrane properties and GABA responses after zinc washout. (d) Plot of peak GABA-evoked conductance increase (left ordinate, ●) and resting input conductance (right ordinate, ○) against time. Zinc was applied at time zero and washed out after 32 min. The onset of the GABA enhancement was closely mirrored by the fall in input conductance.

Inhibition of GABA responses by zinc: a role for GABA_A receptor differentiation?

On the basis of our original observations, GABA responses recorded from rat SCG were assumed to be resistant to the actions of zinc. However, the present data from dissociated cultures of foetal ganglionic neurones clearly resolved an inhibition by zinc of the GABA response. We analysed this apparent anomaly by choosing to study cultured and intact ganglionic neurones from rats of different ages, to decide if the zinc inhibitory action was dependent on neuronal age or merely a susceptibility developed only by cultured neurones. The data indicated that zinc is an effective antagonist of foetal or young post-natal GABA responses whether recorded from intact or cultured neurones, but as the neurones mature, the GABA response becomes apparently less susceptible to zinc inhibition and eventually may become resistant. The latter is also independent of whether or not the neurones are cultured or resident in an intact preparation. This suggested that the

GABA_A-receptor may undergo a subtle modification with time, rendering the receptor less susceptible to zinc inhibition, even though it apparently still possesses a sensitivity to bicuculline and barbiturates (unpublished observations).

Alternatively, zinc may be resolving a different population of GABA receptors which are expressed preferentially at a later stage in neuronal development. At present, these two possibilities are indistinguishable from our experimental evidence.

The discovery of heterogeneity in the GABA_A-receptor subunits (Levitan *et al.*, 1988; Wisden *et al.*, 1988; Pritchett *et al.*, 1989), particularly the α subunit, with regard to the age of the animal and distribution within the CNS, suggests an intriguing explanation for zinc inhibition of GABA responses. The α_3 subunit seems prominent in the CNS of young calves, whereas α_1 and α_2 do not show much variation with age (Levitan *et al.*, 1988). If a similar situation occurs with other species of neural tissue (cf. Pritchett *et al.*, 1989; Fuchs & Sieghart, 1989; Lolait *et al.*, 1989), then we might predict that a GABA receptor of subunit composition $\alpha_3\beta$ would be more sensitive to zinc compared to $\alpha_1\beta$ or $\alpha_2\beta$ which should be relatively insensitive to zinc. This does not discount the possibility that heterogeneity in other subunits, e.g., β or γ may also be important in conferring a sensitivity on the GABA_A-receptor to zinc block.

Previous studies have observed many different effects of zinc on GABA responses, including a competitive inhibition on frog dorsal root ganglion neurones (Akaike *et al.*, 1987). This competitive-type inhibition was not observed in the present experiments where the zinc block was insurmountable when the agonist concentration was raised. These mature amphibian neurones clearly respond differently to zinc compared to mature mammalian ganglia. In foetal mouse hippocampal neurones, zinc produced a non-competitive blockade of GABA responses (Westbrook & Mayer, 1987) at similar concentrations to those used in the present study. The block was also apparently voltage-independent over the range $+40$ to -120 mV, which would support our conclusion from current-voltage plots, that zinc is a voltage-independent blocker of the GABA_A-receptor complex on sympathetic neurones (cf. Figure 3b).

The non-competitive depression of the sympathetic GABA response by zinc, observed in our study, is likely to be a genuine effect on the receptor complex (preliminary observations with fluctuation analysis, suggest that zinc reduces the probability of opening (P_o) for the GABA channel, rather than the single channel conductance) and not due to complexation of GABA by zinc for two reasons: firstly, the dose-response curves do not match those expected following a theoretical consideration of various complexation models. These predict a far greater depression at low agonist doses and little if any at higher agonist concentrations (Smart & Constanti, 1982). Secondly, the minimal inhibition of GABA responses in some adult neuronal tissues and the apparent enhancement in pyriform cortex also argue against any role for complexation masquerading as a genuine inhibition. One other study on vertebrate neurones has also observed a block by zinc of GABA responses, monitored as a reduction in the GABA-induced depolarization of cat primary afferent nerve terminals (Curtis & Gynther, 1987). Interestingly, other divalent cations including cobalt, nickel and cadmium, reproduced the effect of zinc on the spinal afferent. However, in the same preparation, the GABA-induced depression in the firing rate of spinal interneurons was completely unaffected by these cations (zinc was apparently untested; Curtis & Gynther, 1987). Apart from these studies, zinc has been shown to have either no effect on GABA responses or to induce an enhancement. Hori *et al.* (1987) observed little effect of zinc on ionophoretic GABA responses recorded from pyriform cortex slices. However, their incubation time for zinc was quite short (10 min) and our present study, also in pyriform cortex slices, indicates that an incubation time of at least 10–20 min is required before the apparent enhancement effect of zinc is observed. Wright (1984)

Figure 10 Effect of zinc on the reversal level of GABA responses. (a) Control (upper panel) ionophoretically-evoked GABA responses (90 nA, 10 s) recorded from an olfactory neurone held at different membrane potentials (−80 to −50 mV). Negative current pulses (−0.47 nA, 80 ms, 1 Hz) were applied throughout, to monitor membrane resistance. The biphasic character of the response was revealed at −70 mV, while at −50 mV, both early and late phases were clearly hyperpolarizing. (Lower panel) corresponding GABA responses obtained after 20 min in 300 μM zinc. (b) Plot of GABA response amplitude against membrane potential for same cell as in (a). Responses were measured at latencies of 3 (●) and 10 s (▲) in control solution, and 2 (○) and 10 s (△) after 22 min in zinc. The implied reversal level of the late depolarization was shifted from −60 to −50 mV in the presence of zinc.

also found that zinc did not affect GABA responses in the rat cortex *in vivo*, although zinc was applied ionophoretically for only 1 min.

Enhancement of GABA responses by zinc: a role for dendritic leak conductances?

We previously proposed that the enhancement of GABA responses in cortical neurones by zinc was due to a specific modulatory action of zinc at the postsynaptic GABA_A-receptor (Smart & Constanti, 1983). However, in the present study, the enhancement was reproduced by barium (and to some extent by cadmium) which requires a different, more general explanation. All of these divalent cations depolarized and reduced the resting input conductance. The latter effect persisted even when the membrane potential was manually 'clamped', although the identity of the affected ionic component is unresolved. The reduction of the leak conductance by divalent cations, coupled with the biphasic nature of the GABA response (see below) could underlie the apparent GABA enhancement, particularly since the delay in development of a clear enhancement (sometimes up to 30 min for zinc) was always temporally correlated with the change in resting conductance.

Biphasic (Cl[−] dependent) responses to GABA have been recorded on a wide variety of central neurones (Alger & Nicoll, 1982; Scharfman & Sarvey, 1987; Thomson, 1988;

Connors *et al.*, 1988). The early fast hyperpolarizing response probably reflects a somatic action of GABA, while the later depolarizing component reflects activation of dendritic receptors. The marked difference in the reversal level of the two response types is then thought to reflect an actively maintained difference in transmembrane Cl[−] gradient in the dendrites, compared with the soma (Misgeld *et al.*, 1986; Thompson *et al.*, 1988). The reversal levels determined for these biphasic components (−76 and −60 mV, respectively) are very similar to those measured for comparable chloride-mediated GABA responses in hippocampal cells (Newberry & Nicoll, 1985; Misgeld *et al.*, 1986). Zinc and barium produced a consistent positive shift in the apparent reversal level of the late depolarization, which became the dominant component of the response. Some increase in the amplitude of the potential changes induced by GABA (particularly dendritic) might be expected simply from the zinc-induced decrease in the resting conductance (Ginsborg, 1967). However, the enhanced conductance increases measured during GABA responses, could only be explained if an extra component of conductance was now being added to the total input conductance measured in the soma. It is probable that the ionophoretic GABA pipette, positioned close to the intracellular (somatic) recording electrode, was releasing GABA onto somatic and proximal dendritic regions (bath-applied GABA would presumably access all sensitive regions simultaneously). If zinc or barium were inactivating a component of the resting conductance located on dendritic membranes, then this could allow a

Figure 11 Enhancement of GABA responses by barium (Ba). (a) Two ionophoretic responses to GABA (0 nA = removal of retaining current, and 10 nA, 10 s) were applied to an olfactory neurone maintained at -70 mV throughout. Upper records, control responses, then responses elicited after 5 or 15 min in barium solution; lower records were obtained 45 min after barium washout. Barium induced a dramatic increase in cell input resistance and a clear (reversible) enhancement of GABA effects (injected current pulses = -0.45 nA, 150 ms, 1 Hz). (b) Peak GABA-evoked conductance increase (left ordinate, ▲) and resting conductance (right ordinate, △) plotted against time. Barium (300μ M) was applied for 20 min from time zero. The GABA response enhancement and change in input conductance induced by barium followed a similar time course.

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greater proportion of the dendritic GABA response to be resolved back in the soma. A blocking effect of barium on resting dendritic K^+ conductances has been demonstrated by Redman *et al.* (1987) in sympathetic ganglia. Interestingly, Newberry & Nicoll (1985) also observed an enhancement of ionophoretic (somatic) GABA responses on hippocampal CA1 cells in the presence of barium (1 mM), although the underlying GABA conductance was not measured.

The initial enhancing effect of cadmium on the adult cortical GABA response could also be explained by an action on the resting input conductance. However, at later times, membrane conductance increased, presumably diminishing the contribution of dendritic GABA conductances to the total response, which would then be manifest as an inhibition.

A physiological role for zinc?

Zinc is known to be preferentially concentrated in specific neuronal structures including the hippocampus and cortex (Crawford & Connor, 1972; Donaldson *et al.*, 1973; Friedman & Price, 1984). Zinc can also be taken up into neuronal tissue (Wolf *et al.*, 1984; Wensink *et al.*, 1988) and high concentrations have been observed in nerve terminals, particularly in the hippocampal mossy fibre terminations (Ibata & Otsuka, 1969; Perez-Clausell & Danscher, 1985) and also within terminal boutons in many other regions of the telencephalon (Perez-Clausell & Danscher, 1985). Electrical or chemical stimulation of the mossy fibres has revealed the presence of a calcium-dependent 'releasable' pool of zinc (Assaf & Chung, 1984; Howell *et al.*, 1984; Charlton *et al.*, 1985) supporting the suggestion that zinc may be released during 'normal' neurotransmission playing a physiological/neuromodulatory role.

Our data indicate that if zinc does interact with the GABA_A-receptor *in vivo*, then this is more likely to occur in young 'immature' neurones. Recent studies suggest that neurotransmitters including GABA and glutamate, may influence the growth and development of nerve fibres (Mattson, 1988), but it remains to be seen if zinc and GABA can indeed modulate neuronal development and synaptic plasticity.

In conclusion, it is suggested that the vertebrate GABA_A-receptor may undergo a subtle modification with time, rendering the receptor less sensitive to zinc. In addition, agents which block dendritic leak channels may actually enhance the recorded ligand-gated conductances when these conductances are spatially spread from the soma to the dendrites.

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