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$GABA_A$ receptors are differentially sensitive to zinc: dependence on subunit composition

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$GABA_A$ receptors with different subunit composition, were expressed in kidney cells and studied by whole cell recording. Expressed $GABA_A$ receptors were differentially sensitive to inhibition by zinc; receptors which lacked the γ subunit were inhibited by zinc. Embryonic neurones also exhibited zinc-sensitive GABA responses, in contrast to adult neurones. This developmentally-sensitive aspect of $GABA_A$ receptor pharmacology may be partly dependent on expression of the γ subunit.

Keywords: $GABA_A$ receptor; zinc; cloned GABA receptors; GABA receptor subunits; hippocampus; sympathetic ganglia; benzodiazepines; patch clamp

Introduction Zinc is a γ-aminobutyric acid (GABA) antagonist on cultured embryonic neuronal $GABA_A$ receptors (Westbrook & Mayer, 1987; Smart & Constanti, 1990). A comparison of embryonic and adult neurones, demonstrated that zinc inhibition of $GABA_A$ responses was correlated with the age of the neurones (reviewed in Smart, 1990). Embryonic neuronal GABA responses were consistently antagonized by zinc in a non-competitive manner, whereas adult neuronal responses were less sensitive and frequently insensitive to zinc antagonism (Smart & Constanti, 1990; Smart, 1990).

The application of cDNA cloning techniques to $GABA_A$ receptors has revealed a variety of different protein subunits (α, β, γ and δ) and also numerous subtypes of individual subunits (e.g. Verdoorn *et al.*, 1990). Functional $GABA_A$ receptors can be expressed in either *Xenopus* oocytes or mammalian cell lines using previously incorporated cRNAs or cDNAs. Homo-oligomeric GABA receptors can be formed from just one species of receptor subunit, or combinations of cRNAs and cDNAs can be used to construct hetero-oligomeric receptors (Malherbe *et al.*, 1990; Verdoorn *et al.*, 1990; Levitan *et al.*, 1988). The developmental aspect of $GABA_A$ receptor pharmacology, revealed by zinc, was conceivably due to different populations of $GABA_A$ receptors existing in embryonic and adult neurones, some sensitive and others insensitive to inhibition by zinc. This could reflect underlying structural differences between adult and embryonic $GABA_A$ receptors, perhaps due to differences in receptor subunit composition or differences in subunit processing.

This study investigated whether the antagonism of $GABA_A$ receptors by zinc was dependent on the receptor subunit composition.

Methods Human kidney cells (A293) were grown in Dulbecco's modified Eagles medium and Hams F12 with 10% foetal calf serum. Mouse $\alpha 1$, $\beta 1$ and $\gamma 2$ subunit cDNAs encoded polypeptides with amino acid sequences virtually identical to equivalent rat $GABA_A$ receptor polypeptides (100%, >99% and >99% respectively) (Kofuji *et al.*, 1991). The murine cDNAs were cloned as EcoR1 fragments into the mammalian expression vector p-Bex1 (British Biotechnology Ltd.). Expression was controlled by the human cytomegalovirus promoter. Kidney cells were transfected by a calcium phosphate technique (Moss *et al.*, 1990) and incubated at 37°C in 95% air:5% CO_2 for 48 h prior to electrophysiology. Cultured embryonic rat sympathetic ganglion (SCG) neurones were prepared as described previously (Smart & Constanti, 1990). Both SCG neurones and kidney cells were superfused at 30°C with 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. Whole-cell recordings were made with patch pipettes (1–5 MΩ) containing (mM): CsCl 120, tetraethylammonium-OH 33, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11 and HEPES 10, pH 7.1. Hippocampal brain slices were cut from young postnatal (P3–11 days; 600 μm thick) or adult ($P > 90$, 400 μm) rats and superfused at 30°C with Krebs containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 25, glucose 11, bubbled with 95% O_2:5% CO_2, pH 7.4. Intracellular recordings were made from CA3 pyramidal neurones with 3M KCl filled microelectrodes.

Results The expression of $GABA_A$ receptors in cell lines formed from different subunit combinations which included an α subunit, generally produced large GABA-induced membrane currents (Moss *et al.*, 1990; Verdoorn *et al.*, 1990). In contrast, receptors lacking an α subunit or homo-oligomeric GABA receptors, produced much smaller GABA-evoked currents making analysis difficult (Levitan *et al.*, 1988; Verdoorn *et al.*, 1990). Therefore, we used two types of $GABA_A$ receptor, formed from either $\alpha 1\beta 1$, or $\alpha 1\beta 1\gamma 2$ subunit combinations. Zinc (50–300 μM) produced virtually no inhibition of the GABA-induced current when whole cell recording from clusters of cells, or single cells, transfected with $\alpha 1\beta 1\gamma 2$ cDNAs (Figure 1a). In contrast, GABA responses mediated by receptors comprising just $\alpha 1\beta 1$ subunits, were clearly inhibited by zinc in a reversible manner (Figure 1b). GABA concentration-response curve analysis revealed a non-competitive inhibition by zinc on $\alpha 1\beta 1$ $GABA_A$ receptors (Figure 1c) with little variation with the agonist concentration (mean inhibition $82 \pm 6\%$; 0.5–10 μM GABA). The IC_{50} for zinc was estimated as 1.5 μM from the zinc inhibition curve (Figure 1d).

The insensitivity to zinc of $GABA_A$ receptors containing the $\gamma 2$ subunit was interesting, since the inclusion of this subunit in the receptor structure also confers a sensitivity to benzodiazepines (BDZ) (Pritchett *et al.*, 1989; Figure 1a). $\alpha 1\beta 1$ expressed receptors are either insensitive to BDZ (Figure 1b), or display an unusual BDZ pharmacology (Pritchett *et al.*, 1989; Malherbe *et al.*, 1990; Moss *et al.*, 1990). We predicted that $GABA_A$ receptors which are insensitive to zinc, should also be sensitive to BDZ and *vice versa*. Accordingly, we investigated the effect of zinc on cultured SCG neurones and pyramidal neurones in hippocampal brain slices. In the same SCG neurones, GABA responses were antagonized by 100 μM zinc and also enhanced by 1–10 μM flurazepam. Zinc still produced a similar percentage inhibition of the GABA response, even in the presence of flurazepam (Figure 2a).

In contrast, GABA responses recorded from young postnatal (P11) and adult (P > 90) hippocampal brain slices were not

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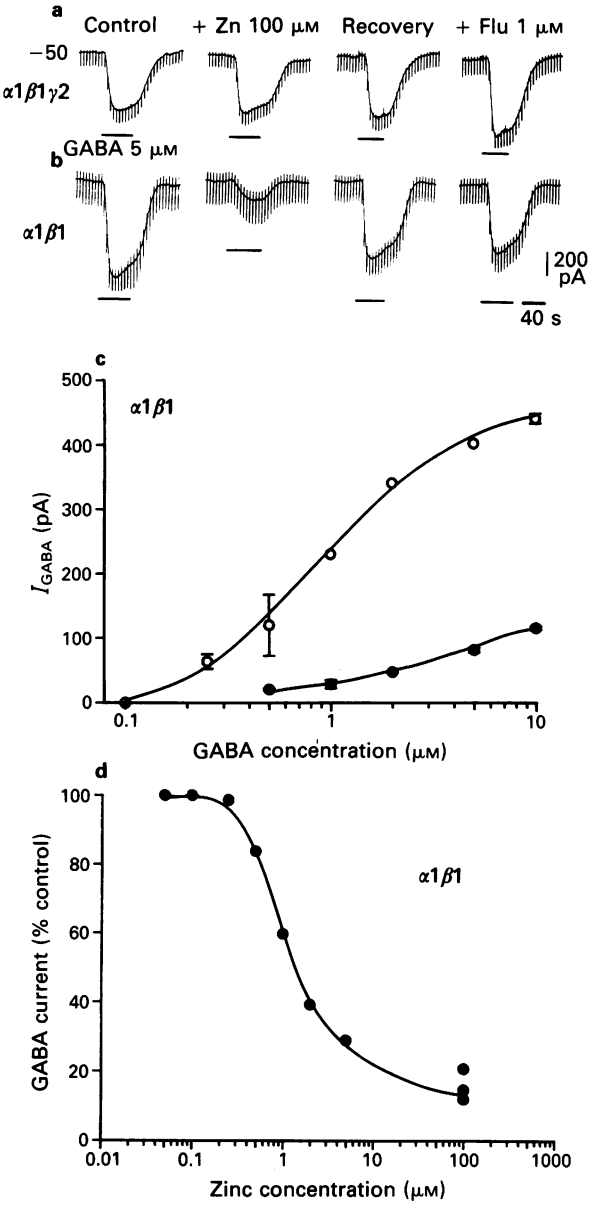

Figure 1 Zinc antagonism of GABA-activated membrane currents mediated by cloned $GABA_A$ receptors of different combinations. (a,b) Whole-cell currents (I_{GABA}) were recorded at −50 mV from cells transfected with $\alpha1\beta1\gamma2$, or $\alpha1\beta1$ GABA cDNAs in the presence and absence of 100 μM zinc, or 1 μM flurazepam (Flu). Brief hyperpolarizing voltage commands were used to monitor membrane conductance (200 ms, −10 mV, 0.2 Hz). GABA 5 μM was bath-applied for the duration indicated by the solid line. (c) Effect of zinc on the GABA concentration-response relationship in $\alpha1\beta1$ transfected cells. GABA responses are plotted as means of 3–5 determinations of the peak I_{GABA} in control (○) and in the presence of 100 μM zinc (●); vertical bars show s.e.mean. (d) Inhibition plot for zinc antagonism of GABA responses mediated by $\alpha1\beta1$ receptors. Peak I_{GABA} was measured to 5 μM GABA in the absence and presence of different concentrations of zinc.

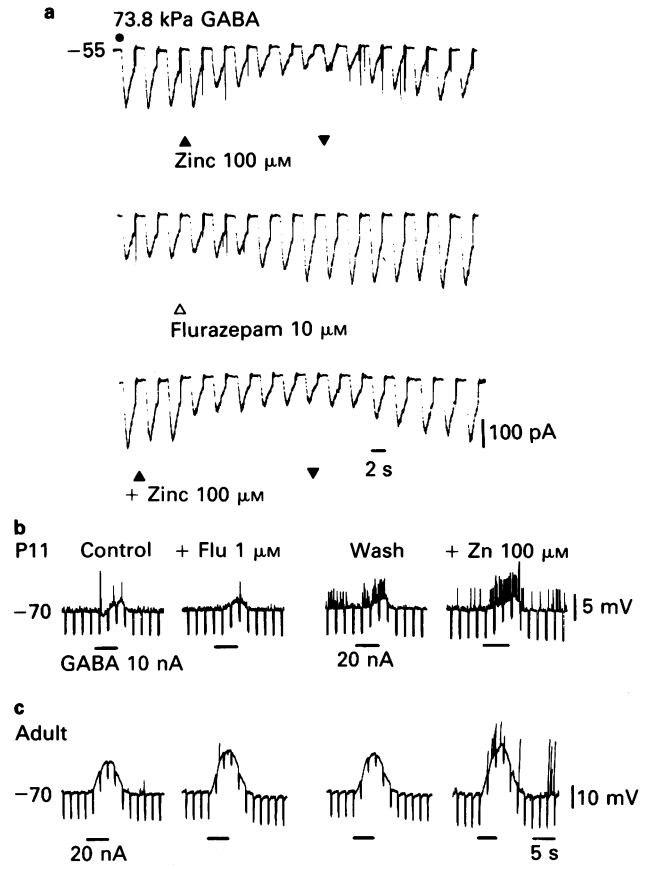

Figure 2 Effect of zinc and flurazepam on GABA-activated membrane currents in vertebrate neurones. (a) Whole-cell I_{GABA} currents were recorded from a cultured superior cervical ganglion neurone (E21) at −55 mV. All three traces represent a continuous recording. GABA responses evoked by pressure application (73.8 kPa; 30 ms; 1 pulse $30\,s^{-1}$) were inhibited by zinc 100 μM. Flurazepam (Flu 10 μM) enhanced GABA responses but subsequent addition of zinc in Flu still produced a comparable inhibition in I_{GABA}. (b,c) GABA-activated depolarizations produced by ionophoretically-applied GABA to hippocampal CA3 neurones. Small hyperpolarizing current pulses (−0.4 nA; 300 ms; 0.5 Hz) monitored membrane resistance. Flu and zinc were bath-applied to young postnatal (P11) (b) and adult (c) neurones. Note Flu produced a clearly enhanced GABA response only in the adult neurones and zinc was ineffective as a GABA antagonist in slices of either age.

blocked by zinc but slightly enhanced, mainly due to a reduction in the resting membrane input conductance (Smart & Constanti, 1990) (Figure 2b). However, in young postnatal slices, flurazepam (0.5–10 μM; $n = 6$) rarely produced any enhancement of the GABA response, but in adult slices, the sensitivity of the GABA response to BDZ became readily apparent (Figure 2c).

Discussion The inhibition of GABA responses by zinc clearly depends on the composition of the $GABA_A$ receptor as receptors possessing a $\gamma2$ subunit are relatively insensitive to blockade by zinc ions. In contrast, expressed $GABA_A$ receptors composed of $\alpha1\beta1$ subunits, were sensitive to zinc inhibition. Since GABA responses from embryonic neurones are routinely zinc-sensitive (Smart & Constanti, 1990; Smart, 1990), the major type of $GABA_A$ receptor present at this stage of neural development might be lacking a $\gamma2$ subunit. Adult and older postnatal forms of the $GABA_A$ receptor would then presumably contain the $\gamma2$, or a functionally equivalent subunit, thereby rendering the receptor insensitive to zinc. However, GABA responses recorded from SCG neurones, retained a sensitivity to both BDZ and zinc in the same cell suggesting that $GABA_A$ receptors with and without the $\gamma2$ subunit were present. Vertebrate neurones are likely to possess a heterogeneous population of $GABA_A$ receptors, and possibly both zinc-sensitive and -insensitive $GABA_A$ receptors may co-exist in the same cell with the balance between these receptor populations varying between embryonic and adult neural tissues.

GABA responses recorded from adult CA3 pyramidal neurones in brain slices were readily enhanced by BDZ and not blocked by zinc, as expected if the major type of $GABA_A$ receptor in adult neural tissues contains the $\gamma2$ subunit; however, our results with young CA3 neurones ($P < 11$) indicated that these GABA responses are apparently insensitive to BDZ (Rovira & Ben-Ari, 1990), and also surprisingly insensitive to zinc blockade. If the $GABA_A$ receptor in young CA3

neurones lacks the $\gamma 2$ subunit, then some susceptibility to zinc antagonism would have been expected.

Zinc sensitivity of the $GABA_A$ receptor is apparently determined by the subunit composition and this, in part, is influenced by the absence of the $\gamma 2$ subunit. However, the results with young hippocampal brain slices suggested other combinations of $GABA_A$ receptor subunits which apparently lack the $\gamma 2$ subunit are also likely to be insensitive to zinc.

The differential pharmacology of the $GABA_A$ receptor protein complex towards zinc, which is apparently related to neuronal development, is a novel finding for this receptor and may be useful in probing the subunit composition of different $GABA_A$ receptor subtypes in many other areas of the CNS.

Note added in proof

Following the original submission of this paper, a study by Draguhn and colleagues (1990, *Neuron.*, **5**, 781–788) using recombinant rat $GABA_A$ receptors also revealed that receptors containing the $\gamma 2$ subunit were relatively less sensitive to inhibition by zinc.

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