Br. J. Pharmac. (1984), **83**, 195–202

Inhibition of GABA uptake potentiates the conductance increase produced by GABA-mimetic compounds on single neurones in isolated olfactory cortex slices of the guinea-pig

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1 Membrane potential and input conductance were recorded in single neurones in slices of guinea-pig olfactory cortex *in vitro*. γ-Aminobutyric acid (GABA) and GABA-mimetic compounds were applied by bath-perfusion. Potency was measured as the concentration required to double the input conductance.

2 The potency of GABA was increased (i.e. the equi-effective concentrations were reduced) by 15.5 ± 2.3 times (mean ± s.e.mean) on reducing external $[Na^+]$ from 144 to 20 mmol l^{-1}, by replacement with Mg^{2+}. Corresponding potency changes for other agonists were $+10.8 \pm 2.5$ for 3-aminopropanesulphonic acid (3-APS); 3.25 ± 1.06 for isoguvacine and 2.43 ± 0.69 for muscimol.

3 Nipecotic acid (0.5 mM) produced the following increases in potency: GABA 2.68 ± 0.82; 3-aminopropanesulphonic acid, 3.11 ± 0.07; isoguvacine, 1.92 ± 0.34; muscimol, 2.24 ± 0.17.

4 The concentration of GABA in the bathing fluid necessary to double input conductance increased with increasing depth of the recording site from the cut surface. The apparent potency fell 10 times for each 60 μm depth increment up to 150 μm. The recording depth also affected the apparent potency of muscimol and 3-APS but to a lesser extent. Reduction of external $[Na^+]$ reduced the depth-dependence of both GABA and 3-APS potency.

5 No clear change in the duration of the recurrent inhibitory postsynaptic conductance could be detected in the presence of 0.5 mmol l^{-1} nipecotic acid.

6 It is suggested that agonist uptake by a Na^+-dependent, nipecotic acid-sensitive mechanism severely attenuates the responses of olfactory neurones to exogenous GABA and to its analogues 3-APS, muscimol and isoguvacine, but has little immediate influence on the duration of the GABA-mediated inhibitory postsynaptic conductance.

Introduction

It is widely thought that the action of the central inhibitory neurotransmitter, γ-aminobutyric acid (GABA), is limited by carrier-mediated uptake from the extracellular fluid into intracellular sites in neurones and possibly glial cells (see, for example, Iversen & Kelly, 1975). However, direct evidence for such a limitation in the central nervous system is still rather sparse. Thus, although there have been several reports that the extracellularly-recorded electrical responses to exogenous GABA are augmented by procedures thought to inhibit the carriers (see Curtis *et al.*, 1976; Lodge *et al.*, 1977; 1978; Brown *et al.*, 1980), only limited information on the effect of carrier-inhibition using intracellular recording is available (Korn & Dingledine, 1982). Further, the evidence that uptake controls the duration of GABA-mediated synaptic inhibition is conflicting and equivocal (compare Lodge *et al.*, 1977; Matthews *et al.*, 1981).

In the present experiments we have tried to assess the influence of uptake on the conductance changes of single neurones in the isolated mammalian olfactory cortex produced by exogenous GABA and also during recurrent inhibition. To do this, we have used

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the two methods of inhibiting uptake previously found to reduce radiolabelled GABA uptake and to increase extracellularly-recorded responses to GABA in this preparation (cf. Brown, *et al.*, 1980): reducing external [Na^+] (Martin & Smith, 1972) and adding nipecotic acid (see Johnston *et al.*, 1976). The results of some of these experiments have been published in abstract form (Galvan & Scholfield, 1978).

Methods

Slices of olfactory cortex (600 μm thick) were obtained from the brains of decapitated guinea-pigs. These were placed in a recording bath at 25°C as described previously (Scholfield, 1981). Intracellular recordings were obtained using glass microelectrodes filled with 4M potassium acetate and coupled to an amplifier equipped with a current injection source to measure input conductance. Agonist responses were measured as the increased input conductance (Scholfield, 1982), calculated by the relationship:

$$G_{agon} = (I/V_{agon}) - (2I/[V_1 + V_2])$$

where I is the amount of constant current passed into the cell during a 180 ms pulse, V_{agon} is the resultant voltage excursion in the presence of agonist and V_1 and V_2 the voltage excursions produced by the same amount of current before adding, and after recovery from, agonist respectively. This method of expressing agonist effects on input conductance excludes any change in the resting conductance by the conditioning agent alone. It assumes a linear current/voltage relationship: this is approximately true for small (about 10 mV) depolarizing excursions from the resting potential of about -75 mV, as used in the present study (see Scholfield, 1978a). The reversal potentials were calculated by interpolation from these current-induced depolarizations and from the slow potential change produced by the agonist (see Brown & Scholfield, 1979).

In many experiments, the distance between the neurone soma (the presumed recording site) and the cut surface of the slice was measured to within ± 3 μm using the scale on the micromanipulator driving the micro-electrode. This was performed by placing the cut surface uppermost and lowering the electrode until it touched the slice as indicated by an increase in the electrode resistance and a few millivolt changes in its potential. Most of the neurone impalements near the cut surface were achieved in thinner slices. The distance between the recording electrode and the pial surface was ignored because preliminary experiments revealed that when the recording electrode was in the middle of the slice, application of GABA to the pial surface only produced very weak responses compared to more intense and prompt effects when the same dose was applied to the cut surface. Thus it would appear that the main route of entry into the slice was via the cut surface. This accords with previous autoradiographs of [^{3}H]-GABA distribution in the slices after short (5 min) incubation times (Brown *et al.*, 1980).

Solutions

Normal Krebs solution had the following composition: (mmol l^{-1}) Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.3, SO_4^{2+} 1.3, PO_4^- 1.2, Cl^- 123, HCO_3^- 25. In experiments using 20 mmol l^{-1} Na^+, either Tris–HCl, $MgCl_2$, LiCl, sucrose or mannitol were substituted for Na^+. These solutions were prepared by adding the appropriate substance to a Krebs solution modified from the one above to contain only 20 mmol l^{-1} Na^+ and (in the case of mannitol and sucrose) 10 mmol l^{-1} Cl^-. Lower Na^+ concentrations depolarized neurones and increased their input conductance. This effect was also observed initially using Tris solution with 20 mmol l^{-1} Na^+ but could be obviated by adding 20 mmol l^{-1} $MgSO_4$. This procedure was ineffective with Li^+ as the Na^+-substituent. The low Na^+ solutions selected for further experimentation contained either 96 mmol l^{-1} $MgCl_2$ (Mg^{2+} solution) or 134 mmol l^{-1} Tris and 20 mmol l^{-1} $MgSO_4$ and enough concentrated HCl to adjust the pH to 7.3 (Tris solution). Low Na^+ solution changed the electrode potential by a few mV; a correction was made for this by passing the solution through the recording chamber in the absence of a slice. Since the agonist concentration-effect curves showed an 'exponential' increase without attaining a clear maximum, the usual ED_{50} was unobtainable. Instead, agonist potency was measured as the dose of agonist required to double the cell's input conductance – that is, to increase the input conductance by the same value as the resting conductance at the start of the experiment (see Scholfield, 1982). Low Na^+ solutions or solutions containing other uptake inhibitors were added 10 min before testing agonists. This was twice the time required for the low Na^+ solution to abolish the action potential, suggesting adequate equilibration. GABA or other agonists were added to the perfusion fluid for 2 to 4 min.

Results

(1) *Responses to GABA*

We used two procedures in these experiments thought to inhibit GABA uptake – reducing the Na concentration of the bathing solution or adding 0.5 mmol l^{-1} nipecotic acid (see Brown *et al.*, 1980).

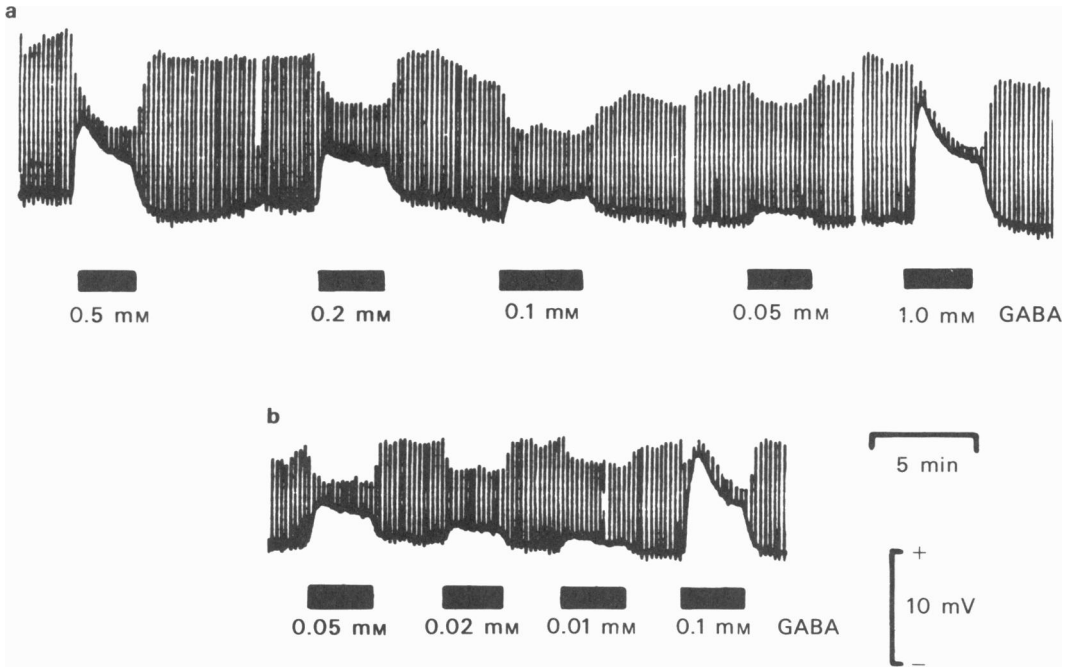

Figure 1 The action of GABA in (a) 144 or (b) 20 mmol l^{-1} Na^+. In (a) the slice was superfused with normal Krebs solution and GABA applied to the bathing solution for 3 min periods (filled bars) at the following doses: 0.5; 0.2; 0.1; 1.0 and 0.05 mmol l^{-1}. The slice was then superfused with a solution containing 20 mmol l^{-1} Na^+ and 96 mmol l^{-1} $MgCl_2$ (b). The resting potential changed from −75 to −73 mV (after correcting for changes in the electrode potential) and GABA was applied at 0.05; 0.02; 0.01 and 0.1 mmol l^{-1} (filled bars). The vertical deflections are depolarizations produced by passing ±0.5 nA current pulses for 130 ms at 0.1 Hz into the neurone, and were used to calculate input conductance changes (see Methods).

Low Na^+ solution Figure 1 shows responses recorded from a neurone in the olfactory cortex slice to varying concentrations of GABA applied to the bathing solution in the presence of either 144 or 20 mmol l^{-1} Na^+ (Mg^{2+} substituted) solutions. In 144 mmol l^{-1} Na^+, GABA depolarized the cell at concentrations of 0.05 mmol l^{-1} upwards, and increased the input conductance as measured by the voltage-deflections produced by +0.5 nA current pulses (see also Brown & Scholfield, 1979). Reduction of Na^+ from 144 to 20 mmol l^{-1} increased the effectiveness of GABA so that the threshold concentration was now 0.01 mmol l^{-1}. The membrane depolarized by about 2 mV in low Na^+ solution with an accompanying increase in the input conductance of the cell (24 ± 5% increase, mean ± s.e.mean). The apparent reversal potential for GABA was about 6 mV less positive, i.e. GABA produced a smaller depolarization for the same conductance change (see Brown & Scholfield, 1979).

Essentially similar results were obtained in 27 other experiments using either Mg^{2+} as the Na^+ substituent and in 3 experiments using Tris-substituted solution. The potency of GABA, measured as the concentration required to double input conductance (see Methods) was increased between 8 and 63 times in Mg^{2+}-substituted solution (mean 15.5 ± 2.4 times : Table 1) and the reversal potential was 8–9 mV more negative. Adding 96 mmol l^{-1} $MgSO_4$ to normal solution did not affect the potency of GABA.

Nipecotic acid Nipecotic acid (0.5 mmol l^{-1}, 7 experiments) also produced a small depolarization (2.0–0.5 mV) and a 21 ± 3% increase in the resting input conductance. This effect was blocked by 10 μM bicuculline. At this concentration of nipecotic acid, GABA potency was increased 2–3 fold (Table 1). In contrast to low Na^+, the reversal potential was unchanged.

Nipecotic acid accelerates the spontaneous release of [^{3}H]-GABA from olfactory cortex slices (Brown *et al.*, 1980), and might potentiate GABA by adding a component of interstitial 'leakage' GABA to that applied exogenously. To test what effect this might have, responses to GABA were measured before and after adding a low concentration of muscimol (1 μM), sufficient to increase input conductance by ~20%.

Table 1 Responses of olfactory cortex neurones to GABA and GABA-mimetic compounds

Test solution (1)	*Agonist* (2)	*No. of determinations* (3)	*Effective concentration of agonist* (mM) *Control* (4)	*Test* (5)	*Test:control* (6)	P (7)
[Na^+] (20 mM)	GABA	28	0.77 ±0.22	0.042 ±0.009	15.51 ± 2.32	<0.001
	3-APS	23	0.019 ±0.005	0.0017 ±0.0007	10.76 ± 2.52	<0.01
	Isoguvacine	3	0.0155 ±0.0036	0.0055 ±0.0012	3.25 ± 1.06	<0.05
	Muscimol	3	0.0040 ±0.0025	0.0024 ±0.0018	2.43 ± 0.69	<0.05
Nipecotic acid (0.5 mM)	GABA	7	0.74 ±0.18	0.39 ±0.13	2.68 ± 0.82	<0.01
	3-APS	4	0.0181 ±0.0170	0.0069 ±0.0042	3.11 ± 0.37	<0.01
	Isoguvacine	4	0.0190 ±0.0073	0.0126 ±0.0066	1.92 ± 0.34	<0.02
	Muscimol	3	0.0062 ±0.0017	0.0028 ±0.0007	2.24 ± 0.17	<0.01
Muscimol (1 μM)	GABA	3	0.72 ±0.49	1.03 ±0.74	0.74 ± 0.03	<0.05

Numbers give mean concentration (± s.e.mean) of agonist required to double neuronal input resistance in normal Krebs solution (control) and in the modified solution indicated in column (1). The ratio in column (6) refers to the mean ratio of effective concentrations in the two solutions determined in single neurones; *P* values show probability that this ratio differs from unity.

When superimposed on muscimol, the apparent potency of GABA was reduced rather than increased, to 0.74 ± 0.03 of the control ($n = 3$).

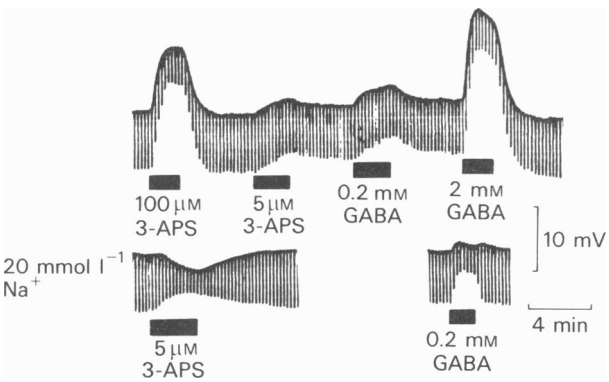

Figure 2 Comparative effects of reducing external [Na^+] on responses of a neurone to 3-aminopropropanesulphonic acid (3-APS) and GABA. Matched responses to the two agonists were first obtained in normal Krebs solution (upper records) and two equi-effective concentrations then administered after reducing external [Na^+] to 20 mM by substitution with Mg^{2+} (see Methods). Note that the input conductance increase produced by both agonists was increased in low Na^+ solution.

(2) *Responses to 3-aminopropanesulphonic acid (3-APS), isoguvacine and muscimol*

These GABA-mimetics appear to be much weaker substrates for the GABA-carriers than GABA itself (see Beart & Johnston, 1973; Olsen *et al.*, 1975; Johnston *et al.*, 1978; Schousboe *et al.*, 1979; Breckenridge *et al.*, 1981; White & Snodgrass, 1983). In agreement with this, 3-APS, at concentrations up to 1 mM, did not appreciably modify [^{3}H]-GABA transport in intact olfactory slices, and the surface depolarization produced by 3-APS (50 μM) was not enhanced by nipecotic acid (Brown *et al.*, 1980). Notwithstanding, in the present experiments low Na^+ or nipecotic acid clearly and consistently enhanced the potency of 3-APS, isoguvacine and muscimol (Table 1). As shown in Figure 2, the conductance changes produced by 3-APS were augmented by about the same amount as those to GABA on switching to a low Na^+ medium. These changes were reversible on returning to normal medium, when tested. (The depolarization produced by 3-APS was clearly diminished in low Na^+ solution in Figure 2 – indeed it reversed to a hyperpolarization. This was occasionally seen with 3-APS and muscimol. It might suggest a component of increased Na^+-conductance in the total response: see Discussion).

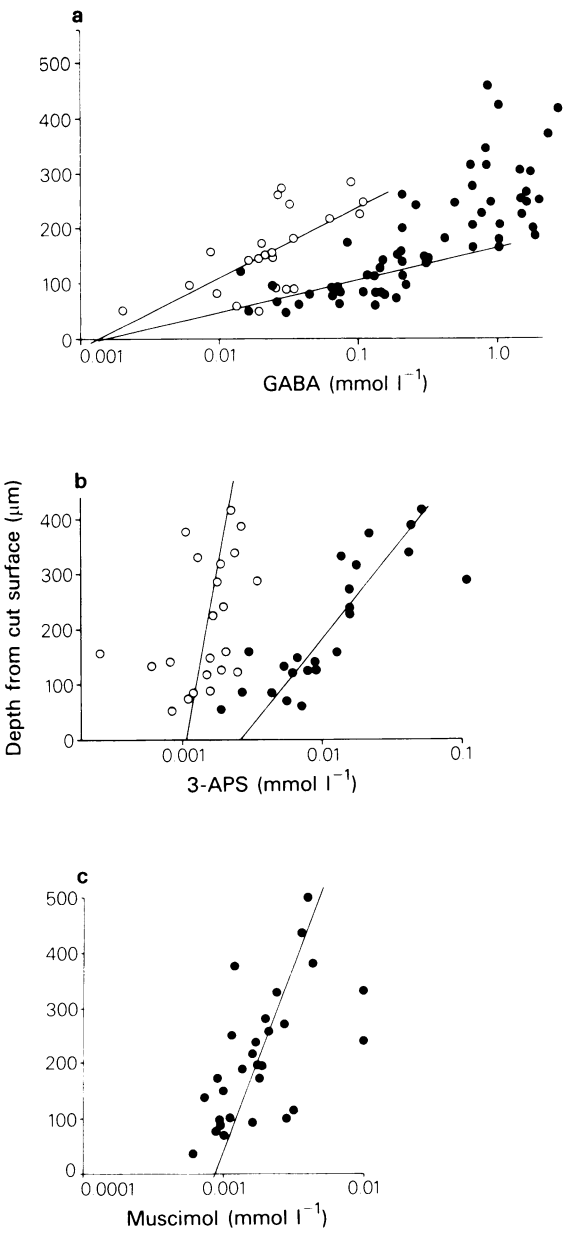

Figure 3 Depth-dependence of apparent sensitivities to (a) GABA, (b) 3-aminopropanesulphonic acid (3-APS) and (c) muscimol. Graphs show the effective concentration of agonist in the bathing fluid (in mmol l^{-1}) required to double neuronal input conductance (see Methods) plotted against the depth of the neurone from the cut surface of the slice (in μm). Each point is a measurement on a single cell. Filled circles: normal Krebs solution; open circles, low Na^+ solution (20 mmol l^{-1} [Na^+], 96 mmol l^{-1} [Mg^{2+}]). Lines are least-squares regressions.

(3) *Relationship between depth of recording site and apparent sensitivity to agonist*

The absolute sensitivity of different neurones to GABA, expressed as the concentration in the bathing fluid required to double the input conductance, varied appreciably in these experiments. This variation appeared to be correlated with the depth of the impaled neurone from the cut surface of the slice, such that the external concentration of GABA had to be increased ten fold for each 60 μm increase in recording depth over the first 150 μm in order to obtain a constant conductance increase (Figure 3a). This depth-dependent diminution in apparent sensitivity to external GABA was reduced by half in low Na^+ solution – i.e. the penetration depth necessitating a ten fold increase in external concentration was increased to 130 μm (open circles in Figure 3a).

The apparent sensitivity to 3-APS showed a similar, though much less severe, tendency to fall with increasing depth of the recording site, with a ten fold increase in external concentration per 300 μm electrode advance (Figure 3b). As with GABA, the depth-dependence was reduced in low Na^+ solution. There was no significant difference (Student's *t* test) in the extrapolated effective concentration at zero depth. Variations in sensitivity to muscimol with increasing penetration depth were less (Figure 3c), corresponding approximately to that for 3-APS in low Na^+ solution.

(4) *Inhibitory postsynaptic conductance*

Electrical stimulation of the lateral olfactory tract generates an excitatory postsynaptic potential, with superimposed spike, followed by a prolonged recurrent inhibitory postsynaptic potential during which the neuronal input conductance is increased (Scholfield, 1978b). As shown in Figure 4, the duration and intensity of this inhibitory postsynaptic conductance was not obviously changed in 0.5 mM nipecotic acid solution. In 5 experiments of this type, the mean half-times for the recovery of the conductance to the initial value after a single stimulus were: controls, 236 ± 64 ms; in 0.5 mM nipecotic acid, 208 ± 6 ms.

Discussion

In previous experiments using an extracellular recording technique, Brown *et al.* (1980) found that nipecotic acid potentiated the depolarization of the isolated olfactory cortex to GABA. This was interpreted to suggest that the action of GABA was inhibited by carrier-mediated transport systems. Similar interpretations have been offered for the potentiation by nipecotic acid of iontophoretically-

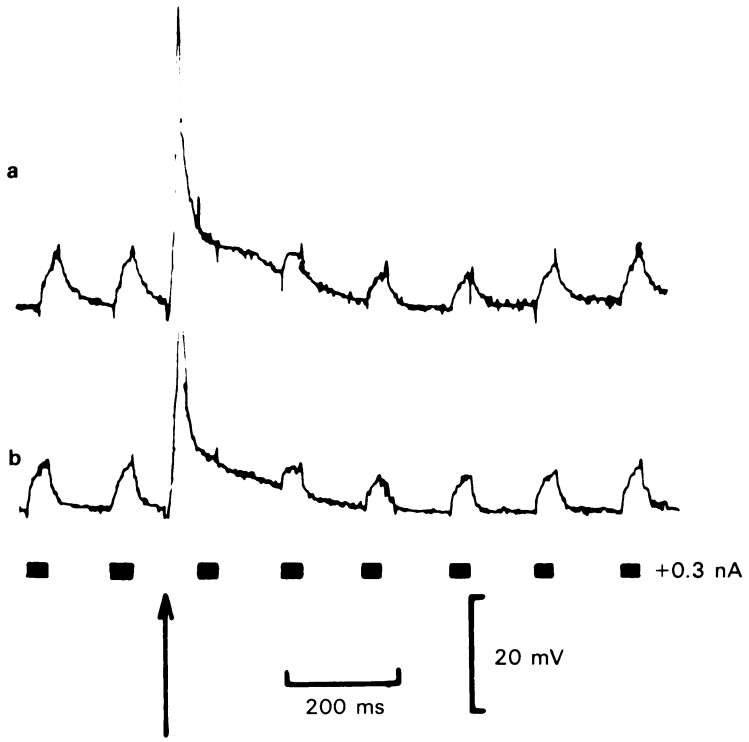

Figure 4 Responses of an olfactory cortex neurone to single stimuli of the lateral olfactory tract (LOT) (at arrow) in (a) normal Krebs solution and (b) after addition of 0.5 mmol l^{-1} (±)-nipecotic acid. During the bars, 30 ms pulses of depolarizing current (+ 0.3 nA) were injected into the cell to monitor input conductance. The LOT stimulus elicited an excitatory postsynaptic potential, with a superimposed action potential (truncated in (b)), followed by a depolarizing recurrent inhibitory postsynaptic potential during which the input conductance was raised (see Scholfield, 1978b).

applied GABA on central neurones *in vivo* (Lodge *et al.*, 1977; 1978). In the present experiments we have confirmed that the intracellularly-recorded conductance change produced by GABA in single olfactory neurones is also potentiated by nipecotic acid and by reducing the Na^+ concentration of the bathing fluid, two procedures which reduce [^{3}H]-GABA uptake by olfactory cortex slices.

However, we have also found that the actions of 3-APS, muscimol and isoguvacine are potentiated by these same procedures. These compounds do not interact strongly with [^{3}H]-GABA uptake in olfactory cortex slices (Brown *et al.*, 1980), and appear to be relatively weak substrates for the GABA carrier(s) in other preparations (Beart & Johnston, 1973; Olsen *et al.*, 1975; Johnston *et al.*, 1978; Breckenridge *et al.*, 1981; White & Snodgrass, 1983; but cf. Agardh & Ehinger, 1982). This raises the question whether the effect of 'carrier-inhibitors' on the potency of these compounds – and, by inference, on the action of GABA itself – is really the result of inhibiting their uptake or of some other effect such as a direct change in receptor sensitivity or a change in background GABA levels consequent upon carrier inhibition.

Changes in receptor sensitivity seem unlikely. Thus, the only directly relevant study (on crustacean muscle: Constanti & Nistri, 1981) suggests that the affinity of GABA for its receptors is reduced in a low Na^+ solution. Likewise, the principal Na^+ substituent used in this study (Mg^{2+}) also reduced the activity of muscimol when added in excess in normal Na^+ solution. Further, the binding affinity of muscimol and GABA for the postsynaptic $GABA_A$ sites seems to be unchanged in Na^+ free solution (Bowery *et al.*, 1981).

An increased background level of GABA is also unlikely to account for the enhanced sensitivity to exogenous GABA-mimetics. Thus, although nipecotic acid did increase cell membrane conductance *per se*, a low concentration of added muscimol sufficient to replicate this action had the effect of reducing the response to exogenous GABA rather than increasing it (Table 1).

Hence it seems most likely that the increased sensitivity to 3-APS, muscimol and isoguvacine in low Na^+ or nipecotic acid solution does, in fact, result from the inhibition of carrier-mediated transport. The reason for this superficially surprising effect is probably that, even though their uptake rate is very low compared with that for GABA, it is sufficiently rapid compared with the rate of inward diffusion to affect their interstitial concentration in these relatively thick tissue slices.

This conclusion receives support from our observation that the concentration of agonist in the bathing fluid necessary to produce a fixed increment in neuronal input conductance had to be increased on recording from cells further from the cut surface of the slice. It seems most reasonable to assume that this results primarily from a gradual reduction in the interstitial concentration of agonist by cellular uptake rather than a change in true sensitivity of the neurones at different depths. The lesser depth-dependence in the apparent effectiveness of muscimol, and the reduction in the depth-dependence of GABA and 3-APS activity in low Na^+ solution, would support this contention.

This has the important consequence that cellular uptake may affect the apparent pharmacological potency, not only of GABA, but also of other analogues with less affinity for the carrier when tested on preparations with restricted diffusional access.

Two further points emerge from these experiments. Firstly, there was no evidence that the duration of synaptic inhibition was limited by GABA uptake as judged by the ineffectiveness of nipecotic acid in prolonging the inhibitory postsynaptic conductance. This accords with previous results from *in vivo* experiments (Curtis *et al.*, 1976; Lodge *et al.*, 1977) although a slight prolongation with nipecotic acid has been found in the isolated hippocampus (Matthews *et al.*, 1981). Secondly, the calculated reversal potential for GABA was more negative in low Na^+ solution suggesting that a Na^+ current contributes to the depolarization (see also Scholfield, 1982). This does not necessarily imply a direct receptor-activated increase in Na^+ conductance: a secondary Na^+ current might perhaps be activated by the depolarization induced by a primary increase in Cl^- conductance (see for example, Stafstrom *et al.*, 1981).

This work was partly supported by a grant from the Medical Research Council.

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(*Received January 26, 1984.*
Revised March 20, 1984.)