Diazepam enhances the action but not the binding of the GABA analog, THIP

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GABA (4-aminobutyric acid) and its bicyclic analog THIP (4,5,6,7-tetrahydroisoxazolo-[4,5-c]-pyridin-3-ol) produced membrane hyperpolarization and increased chloride ion conductance of mouse spinal cord neurons in cell culture. Above 1 nM diazepam enhanced the actions of both GABA and THIP with similar potency and efficacy. Diazepam has been shown to enhance the binding of [3H]GABA to rat brain membranes over similar concentration ranges, with the EC_{50} values for enhancement of [3H]GABA binding and increase in membrane conductance being similar. In contrast, binding of [3H]THIP has been shown to be unaltered by diazepam under a variety of conditions. The possible reasons for such a discrepancy between these electrophysiological and neurochemical results with THIP are discussed.

The inhibitory neurotransmitter, GABA, has a widespread distribution in the central nervous system, and multiple cellular actions of GABA, likely to arise from effects on GABA receptor subclasses, have been identified[11]. To develop GABA agonists with selective actions, certain GABA analogs of restricted conformation have been designed[12,14]. One key compound is THIP, a bicyclic GABA analog, which has potential therapeutic use since it had anticonvulsant and analgesic activity after oral administration to rodents[10,14,16].

Like GABA, THIP produced bicuculline-sensitive inhibition of firing of cat spinal interneurons and displaced [3H]GABA binding to rat brain membranes with moderate potency[7,14,15]. However, important differences between THIP and GABA have been demonstrated. Although THIP was less potent than GABA in depolarizing isolated spinal fibers of the rat, the compound was 20 times stronger than GABA in depression of spinal synaptic activity[2]. GABA usually depolarized apical dendrites of hippocampal CA1 pyramidal cells, while THIP has been shown to elicit hyperpolarizing responses exclusively[1]. Thus, it has been suggested that THIP may selectively activate a subclass of GABA receptors, possibly of synaptic rather than extrasynaptic localization[13].

Diazepam enhanced the effects of synaptically released or exogenous GABA in a variety of central nervous system preparations[9], including cultured mouse and chick spinal cord neurons[6,17]. Diazepam also has been shown to enhance [3H]GABA binding to rat brain membranes[8,18,22,23]. In neurochemical studies, however, anomalous interactions of diazepam and THIP have been documented. Unlike GABA, THIP failed to enhance [3H]diazepam binding at 4 °C and instead antagonized GABA-induced enhancement of benzodiazepine binding[4,13]. While diazepam stimulated the binding of [3H]GABA and [3H]muscimol to rat brain membranes, [3H]THIP binding was unaltered by diazepam under a variety of incubation conditions[23].

Using mouse spinal cord neurons in primary dissociated cell culture, we have investigated the postsynaptic actions of GABA and THIP and the effects of diazepam upon GABA and THIP responses. We report that both amino acids hyperpolarized neurons by increasing chloride ion conductance, the responses were picrotoxin-sensitive and the responses to both amino acids were enhanced with similar potency and to a similar extent by diazepam. We dem-

A1. KAc

GABA 5μM

THIP 200μM

A2. KCl

GABA 5μM

THIP 200μM

B1. GABA

Pre   Picro   Post

B2. THIP

Pre   Picro   Post
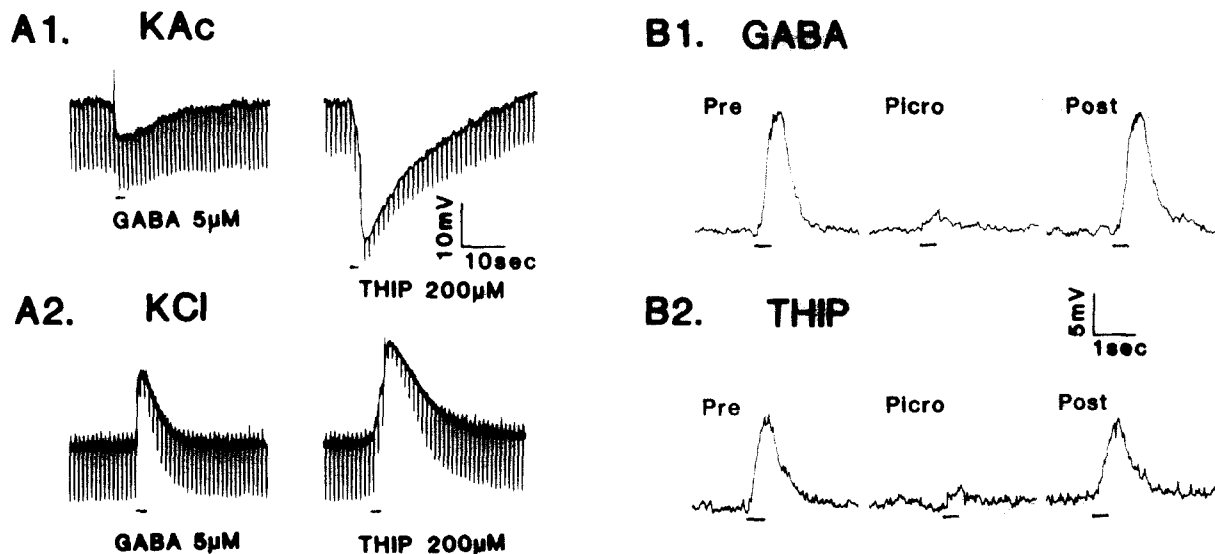

Fig. 1. GABA and THIP responses recorded from spinal cord neurons in cell culture. A: miniperfusion (2 s) with 5 μM GABA or 200 μM THIP increased membrane conductance and evoked hyperpolarizing responses during recordings with 4 M KAc-filled intracellular micropipettes (A₁), and evoked depolarizing responses with 3 M KCl-filled intracellular micropipettes (A₂). Resting membrane potentials were —57 mV and —70 mV, respectively. B: picrotoxin (100 μM) reversibly blocked the effects of iontophoretically applied GABA (8 nA, 400 ms, B₁) and THIP (45 nA, 400 ms, B₂) during recordings with KCl-filled micropipettes. (Membrane hyperpolarized to —90 mV.)

onstrate that enhancement of GABA responses and [³H]GABA binding are similar in dose-dependency and discuss the possible reasons for difference in diazepam action on THIP responses and [³H]THIP binding.

Cell cultures were prepared from spinal cords with attached dorsal root ganglia from 12–14-day-old mouse embryos as described earlier[21]. Cultures were maintained for 5–8 weeks prior to electrophysiological experiments. Spinal cord neurons were penetrated under visual guidance on the modified stage of an inverted phase-contrast microscope, with high impedance (25–40 MΩ) glass micropipettes filled with 4 M potassium acetate (KAc) or 3 M potassium chloride (KCl). Impalements were accepted only if membrane potential was greater than 45 mV and action potentials could be evoked.

Cultures were bathed in a phosphate-buffered saline containing elevated magnesium ions to suppress spontaneous activity (composition in mM: NaCl 143.4, KCl 4.2, CaCl₂ 0.9, MgCl₂ 10.0 and glucose 5.6 in 9.5 mM sodium phosphate buffer at pH 7.35–7.40) and maintained at 33–34 °C for electrophysiological experiments. Using a conventional bridge circuit, simultaneous current injection and membrane potential measurement were possible with a single

recording micropipette; data were recorded on a 6-channel Gould polygraph.

GABA (0.5 M, pH 3.2) or THIP (0.2 M, pH 3.2) were applied iontophoretically using 400 ms duration rectangular current pulses (+0.5–60 nA) at 4-s intervals. GABA or THIP were also applied at known concentrations by miniperfusion using large (2–10 μm) tip diameter micropipettes (0.8 psi ejection pressure, 2 s duration).

Diazepam or its vehicle (0.01% or less dimethylsulfoxide) were applied by miniperfusion (0.2 psi ejection pressure, 30 s duration) from a micropipette positioned 15–100 μm from the soma of the cell under study. Such application of medium containing only 0.01% vehicle did not alter responses to iontophoretically applied GABA (99.7 ± 0.7% control, 6 cells) or THIP (98.6 ± 2.8% control, 6 cells). During assessment of diazepam action upon amino acid responses, cells were impaled with 3 M KCl-containing micropipettes, and GABA or THIP were applied iontophoretically using constant current pulses. GABA and THIP responses of 6–9 mV amplitude were evoked following membrane hyperpolarization to —80 to —90 mV and used for assessment of diazepam actions. Data were accepted only if the amino acid responses returned to control level within 5 min

following removal of the diazepam-containing pipette from the vicinity of the cell under study. In most experiments diazepam effects on GABA and THIP responses were studied on the same cell, with 3 cycles of diazepam application possible.

When KAc recording micropipettes were used, application of GABA or THIP to spinal cord neurons rapidly and reversibly produced membrane hyperpolarization and an increase in membrane conductance (Fig. 1A₁). Use of KCl-containing recording micropipettes allowed chloride ions to enter the cell, changing the equilibrium potential for chloride ions from about —65 mV to —20 mV[19], and both GABA and THIP responses became depolarizing (Fig. 1A₂). At similar amplitudes, responses to THIP were of longer duration than those to GABA, applied either iontophoretically or by miniperfusion for similar durations (Fig. 1A). In 21 cells for which GABA and THIP responses of approximately equal size (about 8 mV) were obtained following 400 ms iontophoretic applications (KCl-filled recording micropipettes), the mean duration at half-maximal amplitude of GABA responses was 0.47 ± 0.02 s and of THIP responses was 0.66 ± 0.02 s ($P < 0.001$, 2-tailed Student's t-test). It has been suggested by others[1] that the greater duration of THIP action may be due to the absence of active uptake mechanisms for this amino acid in neuronal tissue. Indeed, THIP does not inhibit [³H]GABA uptake by minislices of rat cerebral cortex[15], and [³H]THIP is not itself accumulated by such slices (Skerritt, unpublished observations). However, the GABA uptake inhibitor, nipecotic acid (200 μM), failed to increase the duration of responses to GABA recorded from spinal cord neurons in cell culture (unpublished observations). The longer duration of THIP responses could also arise from a greater mean chloride channel open time for THIP than GABA. However, noise analysis studies have actually revealed a shorter mean chloride channel open time for THIP than for GABA[3]. Thus, the basis for the greater duration of THIP responses is uncertain.

Both GABA and THIP responses were virtually abolished in a reversible fashion by the GABA antagonist, picrotoxin (100 μM) (Fig. 1B). The actions of GABA and THIP have been shown also to be reversed by the GABA receptor antagonist, bicuculline, in the rat spinal cord[14].
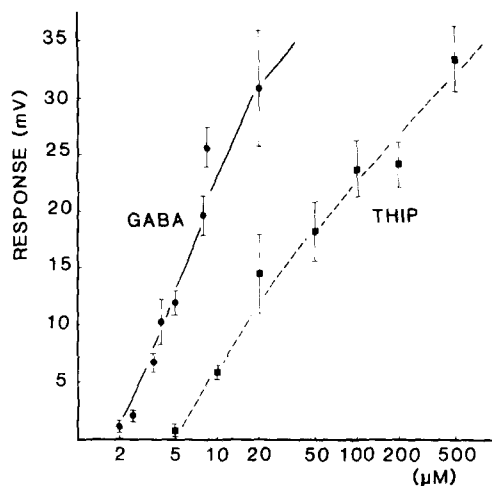
The dose-dependencies of GABA and THIP re-

Fig. 2. Concentration-dependency of GABA and THIP responses. Depolarizing responses were obtained following impalement of cells with 3 M KCl-containing micropipettes and membrane hyperpolarization to —80 to —90 mV. Responses at each concentration are averages of responses on 4–8 cells. Bars indicate ± standard error of the mean (S.E.M.). (Portions of the GABA data are from ref. 19.)

sponses were studied using miniperfusion and recording with 3 M KCl-containing micropipettes (Fig. 2). Threshold for GABA responses was approximately 1–2 μM and for THIP 5 μM. A 10 mV depolarization was produced by approximately 4 μM GABA and 16 μM THIP, a 20 mV depolarization was produced by about 8 μM GABA and 60 μM THIP and a 30 mV depolarization was produced by 19 μM GABA and 350 μM THIP. Thus, over the concentration range studied, GABA was 2.5–25 times more potent than THIP. THIP has been shown to be 3.9-fold less potent than GABA as an inhibitor of [³H]GABA binding to rat brain membranes[6]. THIP has also been shown to be several-fold less potent than GABA in depolarization of ventral roots of neonatal rat spinal cord[2] and depolarization of apical dendrites of CA1 hippocampal pyramidal cells[1]. The dose–response relationship for THIP was thus less steep than that for GABA (Fig. 2), but the slopes of log–log plots of both GABA and THIP dose response-curves are greater than 1.0, suggesting that both GABA and THIP bind cooperatively to receptors.

Diazepam (1–1000 nM) enhanced the effects of iontophoretically applied GABA (Fig. 3, filled circles) and THIP (Fig. 3, open circles), while 0.1 nM diazepam was ineffective. Maximal enhancement for both GABA and THIP occurred at 100 nM and half-
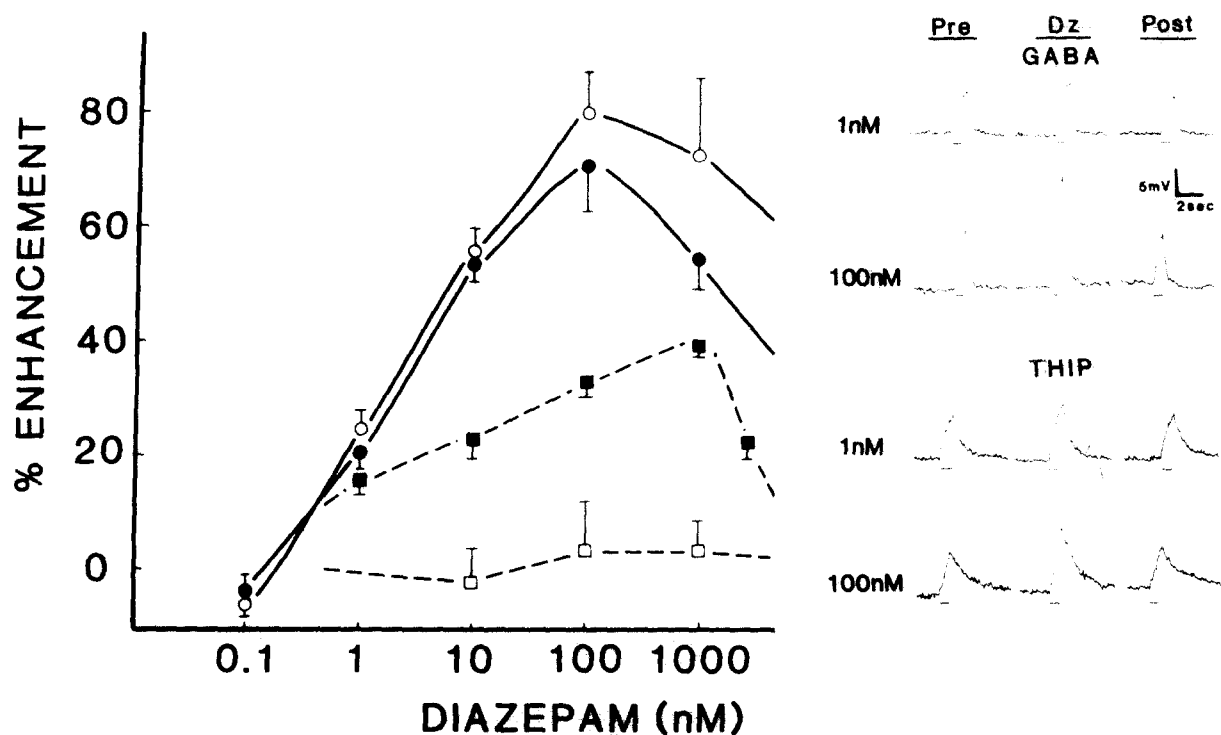
Fig. 3. Concentration-dependency of diazepam effects on GABA and THIP responses and on [³H]GABA and [³H]THIP binding (binding data from ref. 23). 6–15 cells were tested at each diazepam concentration, bars indicate ± S.E.M. Filled circles and squares represent GABA response and binding, respectively, empty circles and squares represent THIP response and binding, respectively. Data shown at right were obtained on one cell (MP = —82 mV) for studies of the effects of 1 nM diazepam on GABA and THIP responses (applied by iontophoresis, KCl recording) and another cell (MP = —80 mV) for 100 nM diazepam.

maximal enhancement at 2.6 ± 0.5 nM (GABA) and 3.6 ± 0.7 nM (THIP) (THIP EC_{50} not significantly different from GABA EC_{50}). The concentration-dependencies of diazepam enhancement of GABA and THIP responses were strikingly similar, and at no diazepam concentration tested was there a statistically significant difference between the degree of enhancement of GABA and of THIP responses (Student's t-test). However, since we did not determine whether or not diazepam had comparable effects on GABA and THIP dose–response curves, we cannot be certain that the mechanism of enhancement was the same.

Diazepam (1–1000 nM) also enhanced the binding of [³H]GABA to fresh, washed synaptosomal membranes from rat brain[22,23], but in contrast, did not enhance [³H]THIP binding under a variety of incubation conditions[24]. The concentration-dependency of enhancement of [³H]GABA binding was quite similar to that of enhancement of GABA responses reported in this study with significant enhancement of 1 nM di-

azepam and 50% maximal enhancement at 4.6 nM. While peak enhancement of GABA responses occurred at lower concentrations than that of [³H]GABA binding, it should be noted that the binding assays illustrated were incubated at 25 °C and the electrophysiological experiments at 33–34 °C; increases in the incubation temperature have been shown to decrease the EC_{50} of concentration–response curve for enhancement of [³H]GABA binding by diazepam[22]. The similarities between the concentration–response relationship for diazepam enhancement of [³H]GABA binding and enhancement of GABA action would suggest that the neurochemical results obtained with diazepam enhancement of [³H]GABA binding are closely related to the effects of diazepam observed in various electrophysiological assays. A good correlation has also been shown between the degree of displacement of benzodiazepine binding in cerebral cortical cultures, and the concentration–dependency of enhancement of GABA actions by diazepam[26]. The disparity between diazepam effects, THIP action and

binding raises the question of whether [³H]THIP and [³H]GABA are binding to the same sites.

Binding studies with [³H]THIP suggest that the ligand labels sites with properties similar to [³H]GABA binding sites. [³H]THIP binding was inhibited by a wide range of GABA analogs with a similar order of potencies to their inhibition of [³H]GABA binding, and [³H]THIP, like [³H]GABA, labeled both higher and lower affinity sites in brain preparations[7,24]. However, the maximal binding capacity of the [³H]THIP sites was considerably lower than that of [³H]GABA, suggesting that [³H]THIP labeled only a subclass of GABA receptors. In addition, the affinities of [³H]THIP for its higher and lower affinity binding sites were greater than those of [³H]GABA, although the potency of THIP on spinal neurons in cell culture was found to be 2.5–25-fold lower than that of GABA. Under standard [³H]GABA binding conditions (Tris-citrate buffer, 2 °C), the equilibrium dissociation constant (K_d) of low affinity [³H]GABA binding to washed synaptosomal rat brain membranes was 822 nM[23], near the threshold for GABA responses observed on spinal cord neurons in cell culture (1–2 μM). However, the K_d value for [³H]THIP was 331 nM, while the threshold for THIP responses was some 15-fold higher (5 μM).

Several explanations are possible for the discrepancy between the neurochemical and electrophysiological results obtained with THIP. Under a variety of conditions diazepam did not enhance the binding of [³H]THIP to the lower affinity site found in brain membranes. It is possible that a THIP receptor of even lower affinity exists. The threshold for THIP actions in spinal cord neurons in cell culture was 5 μM, with larger depolarizations (10 mV) requiring approximately 16 μM THIP. Similarly, for enhancement of benzodiazepine binding (at 30 °C), threshold effects occurred at 10 μM THIP while the EC_{50} value was approximately 40 μM[25]. Other workers[26] have demonstrated the enhancement of the hyperpolarizing actions of a high THIP concentration (50 μM) by 1 μM diazepam in cultured cerebral cortical neurons. If a THIP receptor with a dissociation constant were to exist with a K_d of 20–50 μM, such a site would not be detectable by conventional receptor binding methodologies that use a centrifugation or filtration means of separately bound and unbound radioligand due to the high dissociation rate expected for a site of such low affinity. Whether such a site would be a third binding site for [³H]THIP, or is derived from the lower (331 nM) affinity site when binding is assayed under physiological conditions is unknown. The functions of the subclasses of GABA binding sites labeled by [³H]THIP and unaffected by benzodiazepines is unclear; possibly these sites may be involved in another known action of THIP, such as analgesia[10].

The finding that diazepam enhances THIP actions may be of possible clinical application. While both THIP and diazepam have potent anticonvulsant effects upon acute administration to mice, tolerance develops to the anti-epileptic activity of diazepam[5], and certain side effects (tremor, sedation, ataxia and dizziness) may prevent moderate doses of THIP being used in the clinical setting[20]. Since the enhancement of THIP effects by diazepam could lead to a synergistic rather than purely additive anticonvulsant effect of the two compounds, clinical testing of low dose combinations of THIP and benzodiazepine may prove worthwhile.

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