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Enhancement by GABA of the association rate of picrotoxin and *tert*-butylbicyclophosphorothionate to the rat cloned $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype

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1 We examined how γ-aminobutyric acid (GABA) influences interaction of picrotoxin and *tert*-butylbicyclophosphorothionate (TBPS) with recombinant rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors stably expressed in human embryonic kidney cells (HEK293), as monitored with changes in Cl^- currents measured by the whole-cell patch clamp technique.

2 During application of GABA (5 μM) for 15 s, picrotoxin and TBPS dose-dependently accelerated the decay of inward GABA-induced currents (a holding potential of −60 mV under a symmetrical Cl^- gradient). The drugs, upon preincubation with the receptors, also reduced the initial current amplitude in a preincubation time and concentration-dependent manner. This indicates their interaction with both GABA-bound and resting receptors.

3 The half maximal inhibitory concentration for picrotoxin and TBPS at the beginning of a 15 s GABA (5 μM) pulse was several times greater than that obtained at the end of the pulse. GABA thus appears to enhance picrotoxin and TBPS potency, but only at concentrations leading to occupancy of both high and low affinity GABA sites, i.e., 5 μM. Preincubation of the receptors with the drugs in the presence of GABA at 200 nM, which leads to occupancy of only high affinity GABA sites in the $\alpha1\beta2\gamma2$ subtype, produced no appreciable change in potency of picrotoxin or TBPS. This indicates that they preferentially interact with multiliganded, but not monoliganded receptors, unlike U-93631, a novel ligand to the picrotoxin site, which has higher affinity to both mono- and multiliganded receptors than resting receptors.

4 The time-dependent decay and preincubation time-dependent reduction of initial amplitude of GABA-induced Cl^- currents followed monoexponential time courses, and time constants thus obtained displayed a linear relationship with drug concentration. Analysis of the data using a kinetic model with a single drug site showed that GABA (5 μM) enhanced the association rate for picrotoxin and TBPS nearly 100 fold, but their dissociation rate only 10 fold. The dissociation rate obtained from current recovery from picrotoxin or TBPS block yielded nearly identical values to the above analysis.

5 We conclude that picrotoxin and TBPS interact with both resting and GABA-bound receptors, but their affinity for the latter is about 10 times greater than that for the former, largely due to a markedly increased association rate to the multiliganded receptors (but not monoliganded ones). This and our earlier study with U-93631 improves our understanding of functional coupling between GABA and picrotoxin sites, which appears to be useful in characterizing the mode of interaction for various picrotoxin site ligands.

Keywords: $GABA_A$ receptor; recombinant receptor; picrotoxin; *tert*-butylbicyclophosphorothionate; patch clamp; desensitization; Cl^- channel

Introduction

Picrotoxin and *tert*-butylbicyclophosphorothionate (TBPS) share the same binding site on the $GABA_A$ receptor/channel complex, presumably near the mouth of the Cl^- channel (Squires *et al.*, 1983), and inhibit neuronal GABA-activated Cl^- currents (Bowery *et al.*, 1976; Squires *et al.*, 1983; Ramanjaneyulu & Ticku, 1984; Smart & Constanti, 1986). Recent electrophysiological studies have shown an acceleration of Cl^- current decay (time-dependent block) by the drugs in whole cell patches and a reduction in the channel open probability without altering mean open times in single channel recordings (Hamann *et al.*, 1990; Newland & Cull-Candy, 1992). This supports the hypothesis that picrotoxin and TBPS stabilize the $GABA_A$ receptor in a non-conducting state rather than acting as direct open channel blockers (Takeuchi & Takeuchi, 1969; Barker *et al.*, 1983; Segal & Barker, 1984; Akaike *et al.*, 1985; Smart & Constanti, 1986; Newland & Cull-Candy, 1992). Also, their time-dependent block of the Cl^- channel implies that they interact favourably with GABA-bound receptors in the open state(s), although not as open channel blockers. However, the question of how GABA influences interactions of picrotoxin and TBPS with $GABA_A$ receptors, has not been well understood, partly because most previous studies have been carried out with $GABA_A$ receptors consisting of multiple and functionally diverse subtypes, such as those $GABA_A$ receptors in cultured primary neurones (Barker *et al.*, 1983; Segal & Barker, 1984; Akaike *et al.*, 1985; Yakushiji *et al.*, 1987; Newland & Cull-Candy, 1992), those expressed in *Xenopus* oocytes with injection of isolated brain mRNA (Van Renterghem *et al.*, 1987; Woodward *et al.*, 1992), or those receptors at the invertebrate neuromuscular junction (Takeuchi & Takeuchi, 1969; Constanti, 1978; Smart & Constanti, 1986). In this study, we investigated the effect of GABA on interaction of picrotoxin and TBPS with cloned rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors. This study was also prompted by our recent finding that U-93631 [4-dimethyl-3-t-butylcarboxyl-4,5-dihydro(1,5-a)imidazoquinoxaline], a novel ligand to the picrotoxin site (Dillon *et al.*, 1994), preferentially interacts with mono- or multi-liganded $GABA_A$ receptors over resting ones (Dillon *et al.*, 1993). Our present results demonstrate that picrotoxin and TBPS interact with both resting and GABA-bound receptors,

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but their affinity for the latter is about 10 times greater than that for the former, largely due to a markedly increased association rate to the multiliganded receptors (but not monoliganded ones, unlike U-93631).

Methods

Cloned GABA$_A$ receptors

Rat $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits of the GABA$_A$ receptor were expressed in human embryonic kidney cell lines (HEK293) as described previously (Hamilton *et al.*, 1993). Briefly, the cells were transfected with plasmids containing cDNA and a plasmid encoding G418 resistance. After 2 weeks of selection in 1 mg ml^{-1} G418, resistant cells were assayed by Northern blotting for the ability to synthesize GABA$_A$ receptor mRNAs. Positive cells for appropriate subunits were used for electrophysiology. All studies were conducted on cells expressing the $\alpha 1\beta 2\gamma 2$ receptor configuration.

Electrophysiology

The whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981) was used to study GABA-induced Cl$^-$ currents. Patch pipettes were constructed from borosilicate glass (Kimax-51, Kimble Products, Toledo) pulled (Flaming/Brown, P-80/PC, Sutter Instrument Co., Novato, CA, U.S.A.) and fire-polished to a tip impedence of 0.5 to 2 MΩ when filled with the following pipette solution (in mM): CsCl 140, EGTA 4, HEPES 10, $MgCl_2$ 0.4, pH 7.2. Coverslips containing the cultured cells were transferred to a small chamber (1 ml) on the stage of an inverted light microscope (Nikon), and superfused continuously (2 ml min^{-1}) with the following external solution (in mM): NaCl 125, KCl 5.5, $CaCl_2$ 3.0, $MgCl_2$ 0.8, HEPES-Na 20, dextrose 25, pH 7.3.

Whole-cell currents were recorded with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, U.S.A.) equipped with a CV-4 headstage. A bath headstage (BH-1) was used to compensate for bath potentials. GABA-induced Cl$^-$ currents were monitored on an oscilloscope and stored on a computer using commercially available software (PCLAMP, Axon Instruments). All studies were conducted at room temperature, with the cells voltage-clamped at −60 mV.

Chemicals

GABA and picrotoxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); TBPS was purchased from Research Biochemicals International (Natick, MA, U.S.A.).

Experimental protocol

GABA was dissolved in the external solution (above) and applied to the target cell through a U-tube positioned within 100 μm of the cell. In experiments where preincubation with drugs was required, the cells were bathed in the external solution containing the drugs (picrotoxin or TBPS) at indicated concentrations for 2–20 min (5 min unless noted otherwise). Typically, to monitor GABA response, a GABA pulse (5 μM) was applied before, during and after drug incubation. In experiments examining the kinetic parameters for picrotoxin (PX) and TBPS in the presence of GABA, the GABA pulse applied during incubation was carried out with a mixture of GABA and the incubated drug, so the concentration of the drug was not altered during the pulse.

Data analysis

We examined the effect of picrotoxin and TBPS on peak current and rate of current decay. The time constant for current decay (tau) was obtained by fitting a one-exponential function to time course-current profiles with the aid of a computer software programme (Origin, Microcal Software).

Results

Effect of picrotoxin and TBPS on GABA-induced Cl$^-$ currents

We examined the effects of picrotoxin at various concentrations on GABA (5 μM)-induced Cl$^-$ current in recombinant rat GABA$_A$ receptors of the $\alpha 1\beta 2\gamma 2$ subtype (Figure 1a), using

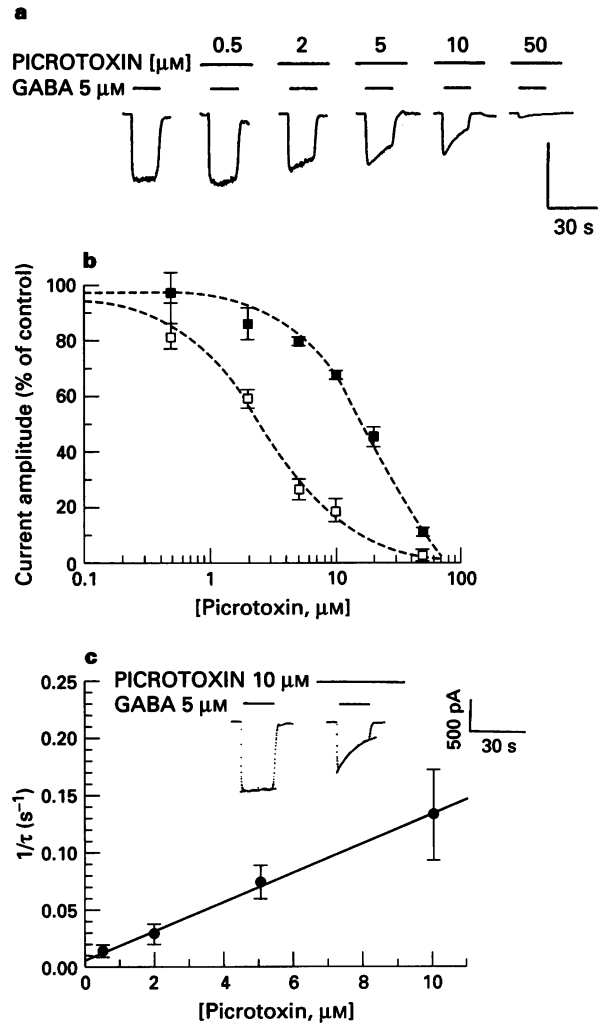


Figure 1 Effect of picrotoxin at various concentrations on GABA-induced Cl$^-$ currents. (a) Picrotoxin decreased the peak amplitude and increased the decay rate of 5 μM GABA-induced Cl$^-$ currents in the $\alpha 1\beta 2\gamma 2$ subtype of GABA$_A$ receptors. Because of the slow current recovery from picrotoxin block at the drug concentrations above 0.5 μM, data were collected from multiple cells, and the differences between cells were adjusted by normalizing to the 5 μM GABA response in each cell. The vertical calibration bar represents 500 pA in the standard cell. (b) Relative changes in the current amplitude at the beginning and end of GABA application were plotted as a function of picrotoxin concentration, then analyzed using a logistic equation (see text). (■) Without GABA; (□) with GABA. Application of GABA for 15 s shifted the IC_{50} for picrotoxin from 17 to 2.4 μM with no effect on the slope. (c) The decay phase of Cl$^-$ currents was fitted with a monoexponential function and the reciprocal of the time constants ($1/\tau$) was plotted as a function of picrotoxin concentration. The solid line represents the best fit to the equation $1/\tau = k_{+1}[PX] + k_{-1}$ with the k_{+1} (the association rate) and the k_{-1} (dissociation rate) values as follows: $k_{-1} = 5.78 \times 10^{-3}\,s^{-1}$; $k_{+1} = 1.31 \times 10^{4}\,M^{-1}s^{-1}$; $K_d = 443$ nM. The inset shows examples of monoexponential fits of the decaying GABA current with and without picrotoxin.

the whole-cell patch clamp technique. The receptors were preincubated with picrotoxin for 5 min and then activated with a 15 s pulse of 5 μM GABA. In the dose-response profiles (Figure 1a), application of picrotoxin was not cumulative; at low picrotoxin concentrations, the cell was washed until the full GABA response was recovered, but at high picrotoxin concentrations a new cell was used because of extremely slow current recovery (see below). Differences in current amplitude between cells were normalized to the 5 μM GABA response. Picrotoxin reduced both the initial peak amplitude of GABA-induced Cl^- currents, and accelerated the current decay (Figure 1a). Changes in the current amplitude at the beginning and the end of GABA application were measured in the presence of picrotoxin at various concentrations, and the data were analyzed with a logistic equation, $I/I_{max} = [\text{picrotoxin}]^n/([\text{picrotoxin}]^n + IC_{50}^n)$, where I is Cl^- current amplitude and n is the slope factor (Figure 1b). Note that the values for the initial current amplitude were obtained upon extrapolation of the decay phase to zero time point using a monoexponential function (see below). From the analysis, we obtained a half maximal inhibitory concentration for picrotoxin of 17 and 2.4 μM at the beginning and the end of GABA pulse, respectively, with a slope factor (n) of 1.1 ± 0.2 and 0.9 ± 0.2, respectively. The increased potency of picrotoxin in the presence of GABA certainly indicates enhancement of picrotoxin interaction with GABA-bound receptors. Furthermore, the reduction of the initial current amplitude appears to reflect its interaction with unliganded resting receptors, because the magnitude of the current reduction was dependent on the duration of incubation time with picrotoxin and its concentration (see below). Similar GABA-dependent changes were observed with TBPS (Figure 2). The half maximal concentration for TBPS was 2.3 μM and 0.8 μM at the beginning and end, respectively, of a 15 s GABA application (Figure 2b). The slope factor 1.2 ± 0.2, was not changed.

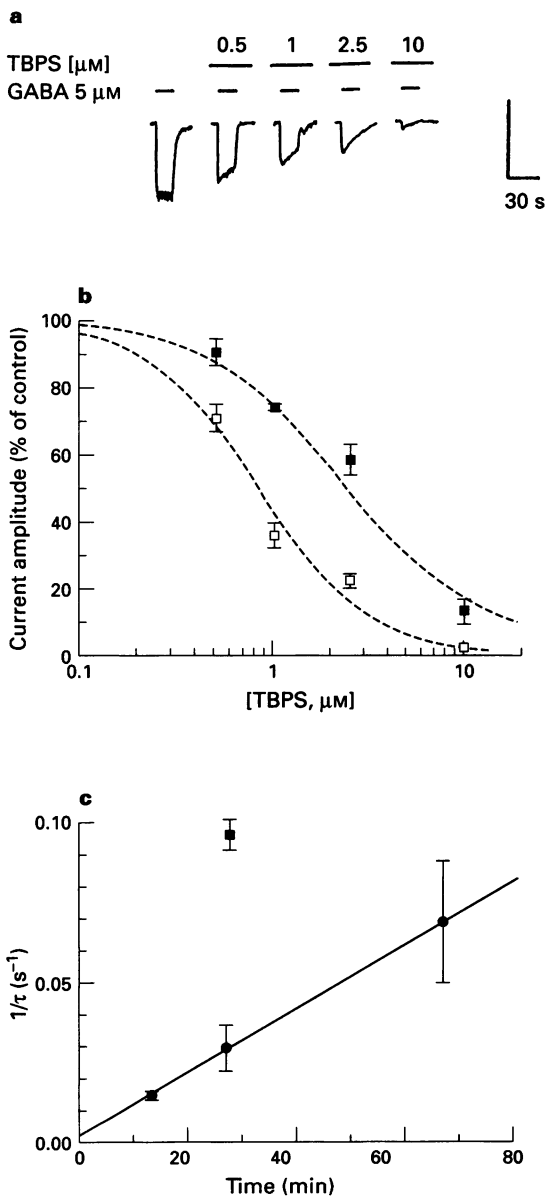


Figure 2 Effects of TBPS at various concentrations on GABA-induced Cl^- currents. The figures represent similar studies to Figure 1, but with TBPS instead of picrotoxin. (a) Current traces with TBPS at various concentrations. (b) Dose-response curves at the beginning (■) and the end (□) of the GABA pulse. Application of GABA for 15 s shifted the IC_{50} for TBPS from 2.4 to 0.8 μM, with no significant change in the slope factor. (c) The decay phase of Cl^- currents was fitted with a monoexponential function and the values of 1/tau were plotted as a function of picrotoxin concentration. The solid line represents the best fit to the equation $1/\text{tau} = k_{+1}[\text{TBPS}] + k_{-1}$, yielding the association and dissociation rates as follows: $k_{-1} = 1.81 \times 10^{-3}\,s^{-1}$; $k_{+1} = 2.71 \times 10^{4}\,M^{-1}s^{-1}$; $K_d = 67$ nM.

GABA at 5 μM, as in the above experiments, occupied both high and low affinity GABA sites of the α1β2γ2 subtype (K_d of 83 and 7100 nM, respectively, Pregenzer *et al.*, 1993), albeit partial. We tested whether occupation of only high affinity GABA sites is sufficient for enhancing picrotoxin potency. To test this hypothesis, the receptors were preincubated with 5 μM picrotoxin or 2.5 μM TBPS in the presence or absence of 200 nM GABA, a concentration at which approximately 70% of high affinity GABA sites were occupied during a 5 min-preincubation period at room temperature. Preincubation with 200 nM GABA did not alter the potency of picrotoxin or TBPS on current induced by subsequent 5 μM GABA. For example, in picrotoxin-treated cells, the initial peak amplitude (after normalization to the control) was reduced to 77 ± 5 and 69 ± 8% with and without 200 nM GABA, respectively, and in TBPS-treated cells, 59 ± 3 and 58 ± 5%, respectively. Likewise, the rate of current decay (time-dependent block) was not altered by preincubation with 200 nM GABA. GABA (5 μM)-induced currents from picrotoxin-treated cells decayed at rates of 12.3 ± 0.7 and 14.7 ± 2.1 s, respectively, with and without 200 nM GABA. Currents from TBPS-treated cells also decayed at comparable rates with and without 200 nM GABA present (10.5 ± 1.4 and 14.4 ± 3.4 s, respectively). Under the same conditions, GABA (200 nM) enhanced the potency of U-93631 (a novel ligand to picrotoxin site) on blocking whole cell Cl^- currents, but did not induce single channel events in cell-attached patches (Dillon *et al.*, 1993). This property differentiates picrotoxin and TBPS from U-93631, another time-dependent blocker of GABA-induced Cl^- currents (Dillon *et al.*, 1993), which preferably interacts with both mono- and multiliganded receptors over unliganded receptors. Overall, our present data suggest that picrotoxin and TBPS preferably interact with multiliganded (probably open channels) over monoliganded or resting receptors.

Effects of GABA on kinetic parameters for picrotoxin and TBPS binding

The decay of GABA-induced Cl^- current accelerated as a function of picrotoxin concentration (Figure 1a) and followed an exponential time course (Figure 1c). A minimal model for picrotoxin interaction with GABA-bound receptors is

$$R^* + D \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} R^*D$$

where R* is the GABA-bound receptor, D is picrotoxin, R*D is the drug-bound, non-conducting receptor, and k_{+1} and k_{-1} are the drug association and dissociation rates, respectively.

This model is justified because the current decay in the presence of 5 μM GABA alone was minimal (see traces in Figure 1a, 1c, $\tau > 200$ s) (Dillon *et al.*, 1993), and the picrotoxin-bound receptors which were produced during resting states display a very slow dissociation rate for picrotoxin (see below) and are not likely to be activated with 5 μM GABA pulses. According to this model, the time constant for current decay (τ) is equal to $1/(k_{+1}[D]+k_{-1})$. The plot of $1/\tau$ vs the concentration of picrotoxin (Figure 1c) was fitted with a linear regression. We obtained a value of 5.78×10^{-3} s^{-1} for k_{-1} (from the y-intercept), 1.31×10^4 M^{-1} s^{-1} for k_{+1} (from the slope), and the dissociation constant ($K_d = k_{-1}/k_{+1}$) of 0.44 μM for picrotoxin in the presence of GABA (5 μM). Similar plots for TBPS yielded k_{-1} of 1.81×10^{-3} s^{-1}, k_{+1} of

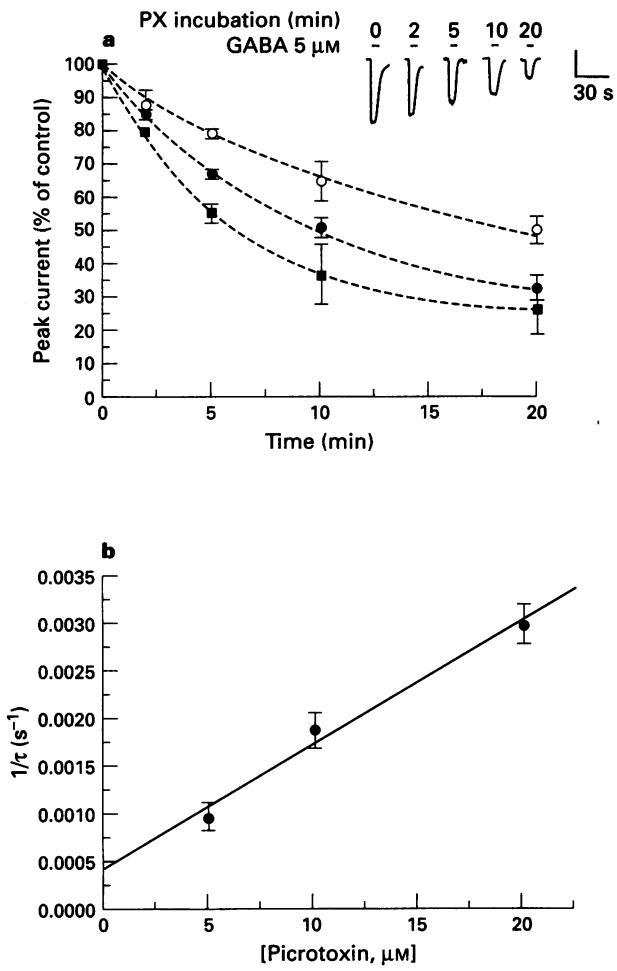


Figure 3 Reduction of the initial amplitude of GABA-induced Cl^- currents by picrotoxin in a preincubation time- and drug concentration-dependent manner. (a) Receptors were incubated for 2, 5, 10 or 20 min with picrotoxin at 5 (○), 10 (●) and 20 μM (■). A 5 μM GABA pulse for 5 s was applied at the end of each incubation period to monitor the change in the initial amplitude of the currents. No picrotoxin was included in the GABA pulse solution and no time-dependent decay was observed. Between the pulses, the cells was washed free of picrotoxin or a new cell was employed. The differences between the cells were normalized as described in Figure 1 legend. The relative changes in the initial current amplitude were plotted as a function of preincubation time at a given picrotoxin concentration, and the plots were fitted with a single exponential function. (b) The reciprocal of the time constants from the monoexponential fitting was plotted as a function of picrotoxin concentrations. The solid line represents $1/\tau = k_1$ [Drug] $+ k_{-1}$ with the association and the dissociation rate for picrotoxin in the absence of GABA: $k_{-1} = 4.2 \times 10^{-4}$ s^{-1}; $k_{+1} = 1.3 \times 10^2$ M^{-1} s^{-1}; $K_d = 3.2$ μM.

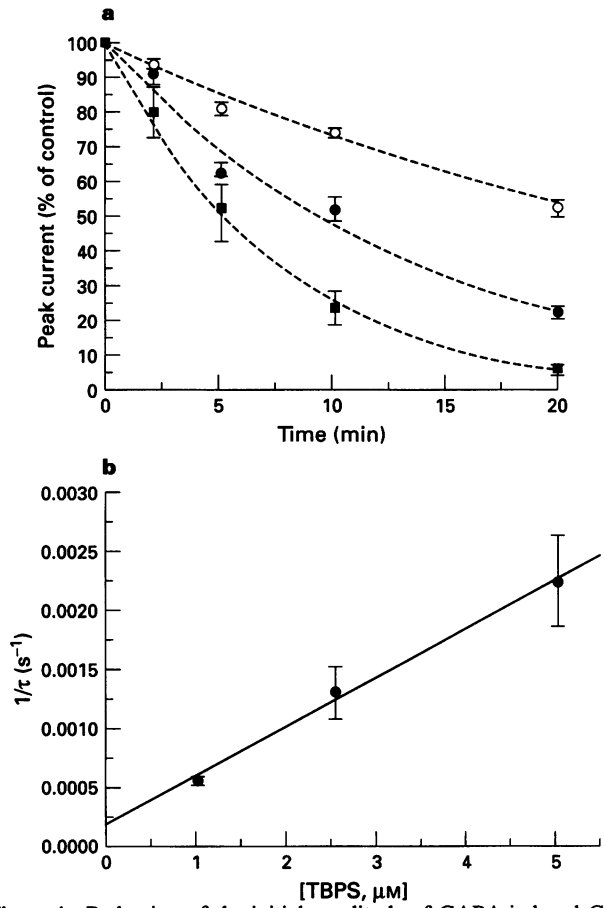


Figure 4 Reduction of the initial amplitude of GABA-induced Cl^- currents by TBPS in a preincubation time- and concentration-dependent manner. The same studies as shown in Figure 3, but with TBPS instead of picrotoxin. (a) Receptors were incubated for 2, 5, 10 or 20 min with TBPS at 1 (○), 2.5 (●) and 5 μM (■). Relative changes in the initial amplitude of GABA-induced Cl^- currents were plotted as a function of preincubation time. The dotted lines represent monoexponential fitting of the data. (b) The reciprocal of the time constants from the monoexponential fitting was plotted as a function of TBPS concentrations. The solid line represents $1/\tau = k_1$ [Drug] $+ k_{-1}$ with the association and the dissociation rate for TBPS in the absence of GABA: $k_{-1} = 1.8 \times 10^{-4}$ s^{-1}; $k_{+1} = 4.2 \times 10^2$ M^{-1} s^{-1}; $K_d = 429$ nM.

Table 1 Effect of GABA on kinetic interactions of picrotoxin and *tert*-butylbicyclophosphorothionate (TBPS) with cloned rat $GABA_A$ receptors of the α1β2γ subtype (see text for details describing how rate constants were determined.)

	Assoc. rate (k_{+1})	*Dissoc. rate* (k_{-1})	K_d (k_{-1}/k_{+1})
Picrotoxin			
No GABA	1.3×10^2 M^{-1} s^{-1}	4.2×10^{-4} s^{-1}	3.2 μM
GABA 5 μM	1.3×10^4 M^{-1} s^{-1}	5.8×10^{-3} s^{-1}	443 nM
TBPS			
No GABA	4.2×10^2 M^{-1} s^{-1}	1.8×10^{-4} s^{-1}	429 nM
GABA 5 μM	2.7×10^4 M^{-1} s^{-1}	1.8×10^{-3} s^{-1}	67 nM

2.71×10^4 M^{-1} s^{-1} and a K_d of 67 nM for TBPS in the presence of 5 μM GABA (Figure 2b and c).

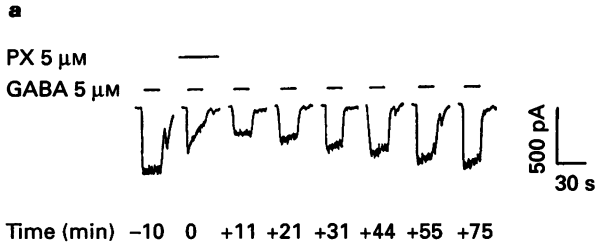
Interaction of picrotoxin and TBPS with $GABA_A$ receptors in the absence of GABA

As noted above, preincubation of the receptors with both picrotoxin and TBPS led to concentration-dependent decreases in the initial current amplitude of GABA-induced currents. The reduction was also dependent on the duration of preincubation period (Figure 3a, inset). For example, picrotoxin at 20 μM reduced the amplitude of the Cl^- currents by 20, 50, 75 and 95% with the preincubation period of 2, 5, 10 and 20 min, respectively. The IC_{50} value, therefore, decreased from much greater than 20 μM with the 2 min preincubation period to approximately 20, 10 and 5 μM as the preincubation period was prolonged to 5, 10 and 20 min, respectively. In order to obtain the reaction rates and the dissociation constant, the relative changes (as normalized to 5 μM GABA response) in the initial current amplitude were plotted as a function of preincubation time of 2, 5, 10 and 20 min (Figure 3a), and the plots were fitted with one exponential function (dotted lines). The time constant decreased linearly as a function of picrotoxin concentration during the preincubation period (Figure 3b). For instance, the τ values were 17 ± 2, 9 ± 0.9, and 6 ± 0.4 min in the presence of picrotoxin at 5, 10 and 20 μM, respectively. This indicates interaction of picrotoxin (and TBPS, see below) with resting receptors in a monophasic manner. Again, one can analyze the data using the same model with a single drug site by simply replacing the term for GABA-bound with resting receptors. Thus, the plot of $1/\tau$ vs picrotoxin concentrations yielded a k_{+1} of 1.3×10^2 M^{-1} s^{-1}, a k_{-1} of 4.2×10^{-4} s^{-1} and a K_d of 3.2 μM for picrotoxin interaction with resting receptors. These results show that GABA markedly enhanced the association rate of picrotoxin to the receptors by 100 fold, and at the same time increased the dissociation rate 14 fold, leading to a 7 fold decrease in the K_d. Similar analysis of TBPS interaction with resting receptors demonstrated comparable effects; i.e., the association rate of TBPS was 64 fold slower with resting receptors than with multiliganded receptors, while the dissociation rate was decreased 10 fold. A summary of the effects of GABA on the kinetic parameters for picrotoxin and TBPS is given in Table 1.

GABA current recovery from picrotoxin and TBPS block

Dissociation rates for picrotoxin and TBPS were also measured from GABA current recovery from picrotoxin and TBPS block (Figure 5). Picrotoxin at 5 μM reduced GABA-induced Cl^- currents by approximately 75% at the end of GABA pulse, including both the initial peak reduction from preincubation and the time-dependent block (Figure 5a). Upon removal of picrotoxin from the superfusing medium, current recovery was monitored with a GABA (5 μM) pulse every 5 min. The amplitude of Cl^- currents progressively increased as a function of time. The plot of fractional recovery vs time (Figure 5b) followed a monoexponential time course (dotted line in Figure 5b) with a time constant of 36.6 min in this cell. The average τ from several cells was 33 ± 4 min. This monoexponential recovery suggests the existence of one population of picrotoxin-bound receptors with a dissociation rate ($1/\tau$) of 5×10^{-4} s^{-1}, which was nearly identical to that observed in the absence of GABA (4×10^{-4} s^{-1}). Current recovery time TBPS (1 μM) also followed a monoexponential time course with a time constant of 38 ± 2 min, which corresponds to a dissociation rate of 4.4×10^{-4} s^{-1}. Again, this value was similar to the value derived using the model (see Figure 4), further strengthening the one-site model.

Previous studies using other $GABA_A$ receptor populations have reported that frequent applications of GABA at high concentrations (0.5 to 2.5 Hz and [GABA] $\geq$ 40 μM, Van Renterghem *et al.*, 1987; Newland & Cull-Candy, 1992)



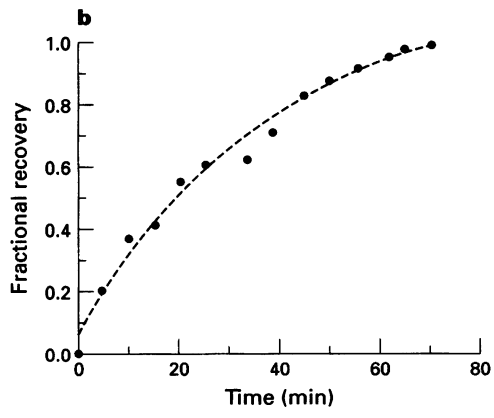


Figure 5 Time course profile for the recovery of GABA-induced Cl^- currents from picrotoxin block. (a) Treatment of the receptors with 5 μM picrotoxin for 5 min reduced Cl^- currents by nearly 70%. Following the treatment, a 5 μM GABA pulse (10 s) was applied every 5 min to monitor the current recovery. (b) Fractional recovery of the current was plotted as a function of time, and the dotted line represents monoexponential fitting of the data. The time constant from this experiment was 36.6 min, and its reciprocal is nearly identical to the dissociation rate obtained from the kinetic analysis of picrotoxin-induced reduction of the initial amplitude of Cl^- currents using the one-site model as described in the legend for Figure 3 and in the text.

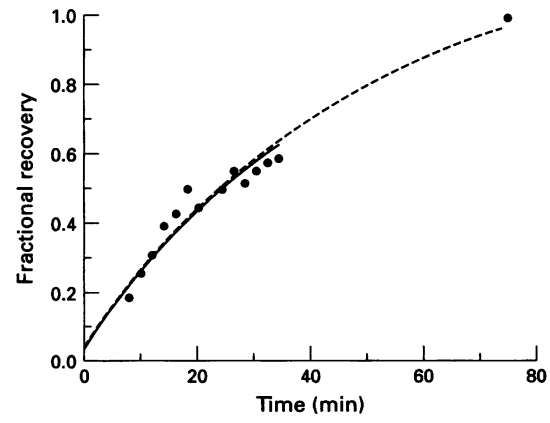


Figure 6 A plot illustrating no use-dependent recovery of GABA-induced Cl^- currents from TBPS block under our experimental conditions. Following treatment with 2 μM TBPS for 5 min, a 5 μM GABA pulse (10 s) was applied every 2 min during the first 36 min period of recovery, and then followed with a final pulse 40 min later. The recovery time course followed an exponential time course during the first 36 min with frequent GABA pulses (solid line), and during the last 40 min without a GABA pulse (dotted line). No appreciable difference was detected in the recovery time constant with ($\tau = 31 \pm 6$ min) or without ($\tau = 38 \pm 2$ min) GABA pulses.

accelerate the recovery of channel activity from picrotoxin or TBPS inhibition (use-dependent recovery). Although the frequency (every 5 min) and the concentration (5 μM) we employed in the current study were well below those used for analysis of use-dependent recovery, we tested whether GABA application at a higher frequency (i.e. every 2 min) affects the dissociation rate for TBPS (Figure 6). This is to ensure not to overestimate the dissociation rate. The fractional increase in Cl^- current with GABA pulses applied every 2 min (as opposed to every 5 min) during the first 30 min was fitted with an exponential function with a time constant of 31 ± 6 min, which was not significantly different from that obtained with GABA pulses applied every 5 min ($\tau = 38 \pm 2$ min, see above). Furthermore, the recovery for the next 40 min without GABA pulses appeared to continue at the same rate, because the final recovery point (70 min from the beginning) reached the level predicted with the same exponential function for the first 30 min (dotted line in Figure 6). It appears that GABA (5 μM) applications at the frequency we used here did not appreciably facilitate the dissociation of TBPS from the $\alpha 1\beta 2\gamma 2$ receptor, which was probably in an inactivated state.

Discussion

In this study, we examined interactions of picrotoxin and TBPS with cloned $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors by investigating their action on GABA-induced Cl^- currents. The drugs, upon preincubation with the receptors, reduced the initial current amplitude, and also produced a time-dependent block of the currents during GABA application. The time (or preincubation time)- and concentration-dependency for the two modes of drug action indicate that picrotoxin and TBPS interact with both resting and GABA-bound receptors. Analysis of the data using a model with a single drug site (see the Results section) revealed that their association rate for GABA-bound receptors was about 100 times (100 fold for PX, 64 fold for TBPS) greater than that for resting receptors, while the dissociation rate, on the other hand, was increased only about 10 fold in GABA-bound receptors. The dissociation rates were also obtained from current recovery from picrotoxin or TBPS block, and were close to the values obtained with the kinetic model in the absence of GABA. Thus, the net effect of the rate changes was to increase the affinity of picrotoxin or TBPS to GABA-bound receptors by approximately 10 fold. Also, it is apparent from our analysis that the time-dependent block of Cl^- currents by picrotoxin or TBPS could be primarily explained by the large increase in their association rate to the site on GABA-bound receptors. According to the model, the time constant for decay is equal to $1/(k_{+1}[D]+k_{-1})$, and at micromolar concentration of the drugs, the term $k_{+1}[D]$ becomes predominant because of relatively small dissociation rates, even in GABA-bound receptors.

Furthermore, the increase in picrotoxin potency (largely due to the association rate) was observed in the presence of 5 μM GABA, but not 200 nM GABA, a concentration at which only high affinity GABA sites are occupied (by approximately 70%). This indicates a preferable interaction of picrotoxin (and TBPS) with multiple-liganded, but not with monoliganded $GABA_A$ receptors (those with only high affinity sites occupied), and is consistent with the view that picrotoxin and TBPS gain much better access to their binding site, probably when the channel enters the open configuration. Although the drugs displayed greater affinity for GABA-bound open channels, they may not act as open channel blockers, but rather as allosteric modulators. This proposal is supported by the work of others (Barker *et al.*, 1983; Segal & Barker, 1984; Smart & Constanti, 1986; Van Renterghem *et al.*, 1987; Woodward *et al.*, 1992; Newland & Cull-Candy, 1992). In these studies, however, changes in kinetic parameters for picrotoxin or TBPS were not quantitatively evaluated, although enhanced association of picrotoxin or TBPS with $GABA_A$ receptors, cloned or native, has been suggested. Further evaluation of previous results is difficult because the subtype composition of $GABA_A$ receptor preparations has not been defined in these studies; i.e., *Xenopus* oocytes injected with either chick brain mRNA (Van Renterghem *et al.*, 1987) or bovine poly(A)$^+$ RNA (Woodward *et al.*, 1992), mouse cultured spinal neurones (Barker *et al.*, 1983), rat cultured hippocampal neurones (Segal & Barker, 1984), or lobster neuromuscular junction (Smart & Constanti, 1986). Overall, the results appear to be in agreement with the view that picrotoxin and TBPS preferentially interact with multiliganded open $GABA_A$ receptors and stabilize the receptors in a desensitized or closed (inactivated) state.

Recently, we discovered a new ligand for the picrotoxin site, U-93631 ([4-dimethyl-3-*t*-butylcarboxl-4,5-dihydro(1,5-a)imidazoquinoxaline]), which also induced a time-dependent block of GABA-activated Cl^- currents in the $\alpha 1\beta 2\gamma 2$ subtype and competitively displaced [^{35}S]-TBPS binding (Dillon *et al.*, 1993; 1994). However, U-93631 is different from picrotoxin and TBPS in that the drug displayed an enhanced interaction with receptors which were preincubated with 200 nM GABA, and thus largely monoliganded. This suggests that although U-93631 shares overlapping binding domains with picrotoxin and TBPS, it also has a unique domain(s) which seems to be coupled to the high affinity GABA sites.

In summary, picrotoxin and TBPS interact with both resting and GABA-bound receptors (multiliganded ones), but their affinity for the latter is about 10 times greater than that for the former, largely due to a markedly increased association rate to the multiliganded receptors. In particular, their enhanced interaction with only multiliganded, but not with monoliganded $GABA_A$ receptors, differentiates them from a new picrotoxin site ligand, U-93631, which showed a higher affinity for monoliganded and multiliganded receptors as compared to nonliganded receptors. It appears that conformational coupling between the GABA and picrotoxin sites is useful in characterizing various picrotoxin site ligands.

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