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Tracazolate Reveals a Novel Type of Allosteric Interaction with Recombinant γ-Aminobutyric Acid$_A$ Receptors

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ABSTRACT

Tracazolate, a pyrazolopyridine, is an anxiolytic known to interact with γ-aminobutyric acid (GABA)$_A$ receptors, adenosine receptors, and phosphodiesterases. Its anxiolytic effect is thought to be via its interaction with GABA$_A$ receptors. We now report the first detailed pharmacological study examining the effects of tracazolate on a range of recombinant GABA$_A$ receptors expressed in *Xenopus laevis* oocytes. Replacement of the γ2s subunit within the α1β3γ2s receptor with the ϵ subunit caused a dramatic change in the functional response to tracazolate from potentiation to inhibition. The γ2s subunit was not critical for potentiation because α1β3 receptors were also potentiated by tracazolate. γ2/ϵ chimeras revealed a critical N-terminal domain between amino acids 206 and 230 of γ2, governing the nature of this response. Replacement of the β3 subunit with the β1 subunit within α1β3γ2s and α1β3ϵ receptors also revealed selectivity of tracazolate for β3-containing receptors, determined by asparagine at position 265 within transmembrane 2. Replacement of γ2s with γ1 or γ3 revealed a profile intermediate to that of α1β1ϵ and α1β1γ2s. α1β1δ receptors were also potentiated by tracazolate; however, the maximum potentiation of the EC_{20} was much greater than on α1β1γ2. Concentration-response curves to GABA in the presence of tracazolate for α1β1ϵ and α1β1γ2s revealed a concentration-related decrease in maximum current amplitude, but a leftward shift in the EC_{50} only on α1β1γ2. Like α1β1γ2s, GABA concentration-response curves on α1β1δ receptors were shifted to the left with increased maximum responses. Tracazolate has a unique pharmacological profile on recombinant GABA$_A$ receptors: its potency (EC_{50}) is influenced by the nature of the β subunit; but more importantly, its intrinsic efficacy, potentiation, or inhibition is determined by the nature of the third subunit (γ1–3, δ, or ϵ) within the receptor complex.

The γ-Aminobutyric acid type$_A$ (GABA$_A$) receptor is a major inhibitory neurotransmitter receptor in the vertebrate central nervous system. In most neurons, the binding of the neurotransmitter GABA to a GABA$_A$ receptor induces an inward Cl^- current, which results in membrane hyperpolarization and reduced neuronal excitability. This ligand-gated ion channel is a heteromeric complex assembled from a number of different subunits (α1–6, β1–4, γ1–4, δ, ϵ, θ, and π) (for reviews, see Barnard et al., 1998; Whiting, 1999). Evidence suggests that in vivo GABA$_A$ receptors are pentameric complexes of α, β, and γ subunits with a stoichiometry of 2α:2β:1γ (Chang et al., 1996; Farrar et al., 1999). The stoichiometry of receptors containing δ, ϵ, and θ is currently unknown, although evidence suggests that δ and ϵ substitute for a γ subunit (Caruncho and Costa, 1994; Quirk et al., 1995; Whiting et al., 1997), whereas θ replaces a β subunit (Bonnert et al., 1999).

The GABA$_A$ receptor is allosterically modulated by a large number of compounds, including benzodiazepines; general anesthetic agents, such as halothane, barbiturates, and etomidate; and neuroactive steroids (Lambert et al., 1995; Sieghart, 1995; Whiting et al., 1995). For a number of these compounds, studies with recombinant receptors have focused on defining receptor subtype selectivity, the amino acids involved in binding, and the mechanism of action. One chemical class of compounds, which is known to modulate GABA$_A$ receptors, that has received little attention in recent years is the pyrazolopyridines, which include tracazolate, etazolate, and cartazolate (Barnes et al., 1983). Behavioral studies have shown that tracazolate and etazolate possess anxiolytic and anticonvulsant activity (Patel et al., 1985; Young et al., 1987). Compared with the standard benzodiazepine chlordiazepoxide, tracazolate was 2 to 20 times less potent as an anxiolytic, but interestingly displayed a much larger window of separation between the anxiolytic effect and potential side effects (sedation, motor incoordination, and its interaction with ethanol and barbital) (Patel et al., 1985).

Herein, we demonstrate that these compounds, particularly tracazolate, possess unique features, modulating these receptors in an allosteric manner previously undescribed,

S.-A.T. and P.B.W. contributed equally to this work.

ABBREVIATIONS: GABA, γ-aminobutyric acid; MBS, modified Barth's solution; TM, transmembrane; SB-205384, 4-amino-7-hydroxy-2-methyl-5,6,7,8-tetrahydrobenzo [*b*]-thieno[2,3-*b*]pyridine-3-carboxylic acid but-2-ynyl ester.

with dimetrically opposite actions on γ2- and ϵ-containing receptors. In addition the generation of chimeric γ2/ϵ subunits implicates the region equivalent to γ2 residues 206 to 230 in determining the nature of this modulation.

Materials and Methods

Human $GABA_A$ Receptor cDNAs. The cloning and sequencing of human α1, α6, β1, β3, γ1, γ2s, γ3, δ, and ϵ and the construction of the single point mutations β1S265N and β3N265S have been reported previously (Wingrove et al., 1994, 1997; Thompson et al., 1997, 1999a; Whiting et al., 1997, and references therein).

Construction of Chimeric Subunits. Seventeen chimeric γ2/ϵ subunits were constructed of which only the five most informative are described herein (Fig. 8). Unique restriction endonuclease sites were introduced into the wild-type sequences by site-directed mutagenesis as described previously (Wingrove et al., 1994). Restriction fragments were gel-purified and ligated using standard techniques. The integrity of chimeric subunits was confirmed by DNA sequencing using an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

Expression in *Xenopus laevis* Oocytes and Electrophysiological Recordings. Adult female *X. laevis* were anesthetized by immersion in a 0.1% solution of 3-aminobenzoic acid ethylester (pH adjusted to toad housing water with 1 M $NaHCO_3$, pH 7.2–8.0) for 30 to 45 min. Ovary tissue was removed via a small abdominal incision and stage V and VI oocytes were isolated with fine forceps. After mild collagenase treatment to remove follicle cells (type IA, 0.5 mg/ml, for 6 min), the oocyte nuclei were directly injected with 10 to 20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7, filtered through nitrocellulose) containing different combinations of human $GABA_A$ subunit cDNAs engineered into the expression vector pCDM8 or pcDNAI/Amp. The ratio of α:β:γ2s constructs was generally 1:0.5:1, whereas the ratio for α:β was 1:1, for α:β:γ3 was 1:1:1, for α:β:γ1 was 1:1:10, and for αβδ and αβϵ was 1:0.5:3 with 1 corresponding to 6 ng/μl of cDNA. Confirmation that all the subunits injected were being expressed was routinely checked using Zn^{2+}, flunitrazepam, or picrotoxin. Oocytes were maintained at 19–20°C in modified Barth's solution (MBS) consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, at pH 7.5 supplemented with 50 μg/ml gentamicin, 10 μg/ml streptomycin, 10 units/ml penicillin, and 2 mM sodium pyruvate, for up to 6 days. For electrophysiological recordings, oocytes were placed in a 50-μl bath and continually perfused at 4 to 6 ml/min with MBS. Cells were impaled with two 1- to 3-MΩ electrodes containing 2 M KCl and voltage-clamped at −70 mV. In all experiments, drugs were applied in the perfusate until the peak of the response was observed. The magnitude of modulation of GABA-evoked currents by allosteric modulators is critically dependent upon the concentration of GABA used (Parker et al., 1986; Wafford et al., 1994). For this reason the modulatory effect of tracazolate was examined against an EC_{20} concentration of GABA (range $EC_{18–25}$), which was determined for every individual oocyte. In all experiments, except those designed to investigate the direct effect of tracazolate, tracazolate was preapplied for 30 s before being coapplied with the appropriate concentration of GABA. To minimize the effect of receptor desensitization, agonist applications were separated by a period of at least 3 min upon recovery back to baseline. All data were expressed as either a percentage modulation of the GABA EC_{20} value, or as a percentage of the maximal response to GABA. Curves were fitted using a nonlinear square-fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^{n_H}]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n_H is the Hill coefficient. For some receptor subtypes high concentrations of tracazolate or etazolate (e.g., 100 μM etazolate on α1β3γ2s, and 30 μM tracazolate on α6β3γ2s) produced responses substantially smaller than the previous response. In these instances curves were fitted to the concentration before this point. Data are presented as the arithmetic mean ± S.E.M. or geometric mean (−S.E.M., +S.E.M.) from a number (n) of different cells. Differences between means were evaluated by analysis of variance and Student's t test and considered significant if $P < 0.05$.

Solutions and Solvents. GABA 1 M stock was dissolved in MBS, $ZnCl_2$ 1 M stock in 0.2 M HCl, and picrotoxin and tracazolate 100 mM stocks and flumazenil 10 mM stock in 100% dimethyl sulfoxide. The limit of solubility of tracazolate in MBS was 100 μM. The maximum concentration of vehicle (0.1% dimethyl sulfoxide) was without effect. GABA, $ZnCl_2$, picrotoxin, and tracazolate were obtained from Sigma Chemical (St. Louis, MO), whereas the Chemistry Department at Merck Sharp and Dohme (Harlow, Essex, UK) synthesized flumazenil.

Results

Potentiation of α1β1γ2s and α1β3γ2s $GABA_A$ Receptors: Selectivity for β3. GABA EC_{20} responses on α1β1γ2s and α1β3γ2s receptors were potentiated in a concentration-dependent manner by tracazolate and etazolate (Table 1; Fig. 1). As stated above, on some receptor subtypes, high concentrations of tracazolate or etazolate produced responses substantially smaller than the previous response. These data points were not included in the curve fitting and to aid visualization of the data were omitted from the graphs. This decrease in apparent efficacy at high concentrations may be an artifact of the slow application time, inherent with the *X. laevis* oocyte system, which may allow receptor desensitiza-

TABLE 1

Summary of concentration-response data for tracazolate and etazolate potentiation of control GABA EC_{20} responses for wild type and mutant $GABA_A$ receptors

Data for the EC_{50} are the geometric mean (−S.E.M., + S.E.M.) and for the maximum potentiation and Hill coefficient are the arithmetic mean ± S.E.M.

Subunit Combination	EC_{50}	Maximum Potentiation	Hill Coefficient	No. of Oocytes
	μM			
Tracazolate				
α1β1γ2s	13.2 (10.2, 17.2)	168 ± 16	1.54 ± 0.11	4
α1β3γ2s	1.5 (1.2, 1.8)	224 ± 46	1.27 ± 0.12	5
α1β1Ser265Asnγ2s	2.7 (2.4, 2.9)	273 ± 60	1.28 ± 0.12	4
α1β3Asn265Serγ2s	6.8 (5.5, 8.5)	207 ± 24	1.10 ± 0.10	4
α1β3	2.7 (2.0, 3.5)	351 ± 94	1.24 ± 0.13	4
α6β3γ2s	1.1 (0.8, 1.6)	363 ± 44	1.42 ± 0.18	4
α1β1γ1	1.3 (1.1, 1.5)	21.0 ± 5.9	1.8 ± 0.5	4
α1β1γ3	1.4 (1.0, 2.1)	34.7 ± 10.1	1.6 ± 0.7	4
α1β1δ	16.7 (14.6, 19.2)	1368 ± 377	1.4 ± 0.06	5
Etazolate				
α1β1γ2s	8.3 (6.8, 10.2)	315 ± 32	1.25 ± 0.09	4
α1β3γ2s	1.3 (1.1, 1.5)	271 ± 34	1.91 ± 0.47	5

tion to occur during the rising phase of the inward current to GABA, resulting in a truncated response. Other explanations such as channel blockade, however, cannot be eliminated from the present data. The maximum potentiation (fitted to the ascending portion) ranged from between 168 and 315% and is comparable with that seen with both full benzodiazepine agonists (Wafford et al., 1993) and many other nonbenzodiazepine modulators of the $GABA_A$ receptor [e.g., loreclezole (Wafford et al., 1994), pentobarbital (Thompson et al., 1996), and neurosteroids (Lambert et al., 1995)]. No significant direct activation by either tracazolate or etazolate was observed over the concentration range examined (30 nM-100 μM). Interestingly, both compounds displayed a significant 6- to 9-fold selectivity for $\alpha1\beta3\gamma2s$ over $\alpha1\beta1\gamma2s$ receptors. Because structurally and functionally tracazolate and etazolate were similar, further investigations were performed with tracazolate only.

β3 Selectivity Is Conferred by Asparagine 265. Selectivity for $\beta2/3$-containing $GABA_A$ receptors over $\beta1$ has previously been reported for loreclezole (Wingrove et al., 1994), β-carbolines (Stevenson et al., 1995), etomidate (Hill-Venning et al., 1997), furosemide (Thompson et al., 1999a), and mefenamic acid (Halliwell et al., 1999). For all these compounds this selectivity has been shown to be due to a critical asparagine residue at position 264 and 265 (numbering according to mature polypeptide sequence) within TM2 of the $\beta2$ and $\beta3$ subunit. It was logical therefore to see whether this residue also determined the $\beta3$ selectivity of tracazolate. The two mutant β cDNAs (β1Ser265Asn and β3Asn265Ser) were coexpressed with $\alpha1$ and $\gamma2s$ and concentration-response curves to tracazolate constructed (Table 1; Fig. 2). Replacement of Ser265 within the $\beta1$ subunit with Asn (the $\beta3$ counterpart) increased the sensitivity of tracazolate, whereas the opposite mutation (Asn$\beta3$ to Ser) decreased the sensitivity to tracazolate. Hence, the $\beta3$ selectivity of tracazolate is determined by asparagine 265 within the $\beta3$ subunit.

Interaction Is Not via Benzodiazepine Binding Site. Concentration-response curves to tracazolate were also constructed on oocytes expressing $\alpha1\beta3$ and $\alpha6\beta3\gamma2s$ $GABA_A$ receptors (Table 1; Fig. 3). Similar to $\alpha1\beta3\gamma2s$ receptors, control GABA EC_{20} concentrations on $\alpha1\beta3$ and $\alpha6\beta3\gamma2s$ receptors were potentiated by tracazolate. Statistical analysis (analysis of variance) revealed no significant differences in the log EC_{50}, Hill coefficient, or maximum potentiation for $\alpha1\beta3$, $\alpha1\beta3\gamma2s$, or $\alpha6\beta3\gamma2s$. Unlike compounds that interact at the benzodiazepine site, receptors lacking a γ subunit were also potentiated by tracazolate. Replacement of the $\alpha1$ subunit with an $\alpha6$ subunit did not alter the concentration-response curve to tracazolate. Finally, 300 nM flumazenil, a benzodiazepine site antagonist, did not affect the degree of potentiation elicited by 10 μM tracazolate on $\alpha1\beta3\gamma2s$ receptors (198 $\pm$ 42%, $n = 5$ in the absence versus 223 $\pm$ 31%, $n = 4$ in the presence).

Tracazolate Inhibits $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ $GABA_A$ Receptors. In addition to replacing the α and β subunits, we studied the effects of replacing the $\gamma2$ subunit. ϵ-Containing receptors reveal some unusual properties, including a proportion of constitutively active channels, fast desensitization

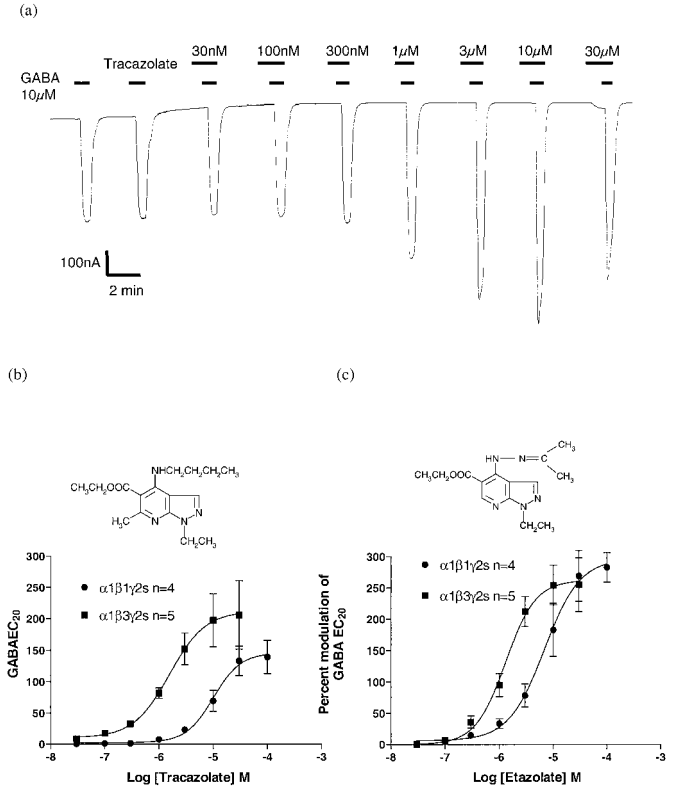


Fig. 1. Tracazolate and etazolate potentiate control GABA EC_{20} responses on $\alpha1\beta1\gamma2s$ and $\alpha1\beta3\gamma2s$ receptors with selectivity for $\alpha1\beta3\gamma2s$ receptors. A, representative trace showing a concentration-response curve to tracazolate on an oocyte expressing $\alpha1\beta3\gamma2s$ receptors. The oocyte was voltage-clamped at -70 mV. The horizontal lines indicate application of tracazolate and GABA. To observe any direct effect and to ensure complete binding, increasing concentrations of tracazolate were applied for 30 s before the coapplication of the GABA EC_{20} concentration and the tracazolate concentration. Note the reduction in response to 30 μM tracazolate compared with 10 μM tracazolate, which was observed in three of the five cells examined. B, modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta1\gamma2s$ (●) and $\alpha1\beta3\gamma2s$ (■) $GABA_A$ receptors. C, modulation of the control GABA EC_{20} response by increasing concentrations of etazolate on oocytes expressing $\alpha1\beta1\gamma2s$ (●) and $\alpha1\beta3\gamma2s$ (■) $GABA_A$ receptors. Data represent the mean $\pm$ S.E.M. from the number of cells indicated from two or more batches of oocytes.

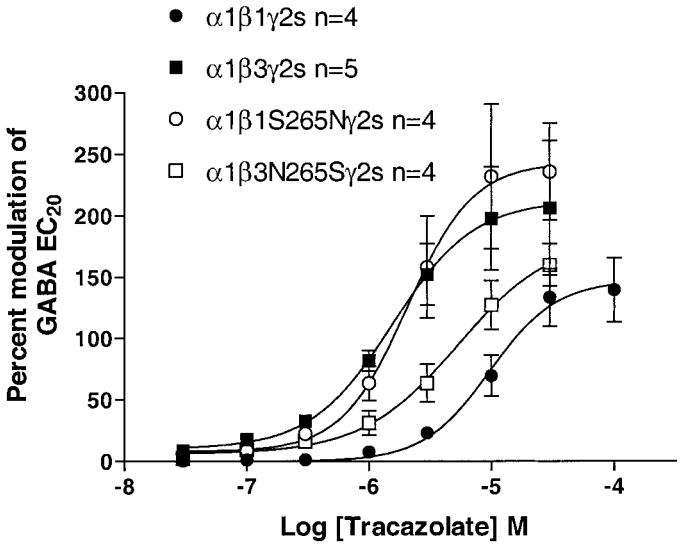


Fig. 2. $\beta3$ selectivity of tracazolate is conferred by asparagine 265. Modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta1\gamma2s$ (●), $\alpha1\beta3\gamma2s$ (■), $\alpha1\beta1$Ser265Asn$\gamma2s$ (○), and $\alpha1\beta3$Asn265Ser$\gamma2s$ (□) $GABA_A$ receptors. Data represent the mean $\pm$ S.E.M. from the number of cells indicated from two or more batches of oocytes.

kinetics, and a transient rebound current on GABA washout (Whiting et al., 1997; Neelands et al., 1999). Unlike the receptor combinations mentioned above, on $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ receptors, tracazolate showed a significant direct effect, with low concentrations producing an inward current, whereas higher concentrations produced an inward current followed by an outward current (Fig. 4, A and B). It has been shown previously that constitutively open channels can be modulated by certain allosteric modulators [e.g., $\beta3$ homomeric receptors (Wooltorton et al., 1997); $\alpha1\beta2L259S\gamma2s$ (Thompson et al., 1999b)] and hence the effect of an allosteric modulator on the constitutively open channels and the GABA-activated channels can be difficult to separate. Further characterization of the direct effect of tracazolate on $\alpha1\beta3\epsilon$ receptors was undertaken in separate experiments. To enable direct comparison of the modulatory effect of tracazolate on $\gamma2$- and ϵ-containing receptors, the application time of tracazolate before coapplication of tracazolate and GABA was kept at 30 s. To further facilitate comparison with the results obtained with $\alpha1\beta3\gamma2s$ and $\alpha1\beta1\gamma2s$ receptors, the inward current to GABA in the presence of tracazolate was normalized with respect to the response evoked by the control GABA EC_{20} response (i.e., the effect of tracazolate on the constitutively open channels was omitted). As illustrated in Fig. 5A the direct effect of tracazolate could take up to 240 s to reach steady state, hence one caveat with the experimental design described above is the introduction of a small degree of error in the measurement of the inward current to GABA. Unlike its effects on $\alpha1\beta3\gamma2$, tracazolate caused inhibition of the control GABA EC_{20} response (Fig. 4; Table 2). The GABA response could be almost completely inhibited, and the IC_{50} values for inhibition by tracazolate on $\alpha1\beta\epsilon$ receptors were similar to the EC_{50} values obtained on $\alpha1\beta\gamma2s$ receptors.

Outward Current to Tracazolate on $\alpha1\beta3\epsilon$ Receptors Is Carried by Cl^- Ions. Concentration-response curves to the direct effects of tracazolate were constructed on $\alpha1\beta3\epsilon$ receptors. As can be observed in Fig. 5A, a small inward current was observed followed by a larger outward current, which increased with increasing concentration up to a maximum response at 10 μM. At high concentrations, the outward current took up to 240 s to reach a plateau followed by a washout period of up to 10 min to reestablish the baseline value; this was hypothesized to be due to block of constitutive activity. The outward current was measured using the peak of the inward current as the start value and normalized to the outward current induced by 100 μM picrotoxin. Picrotoxin (10 μM) has previously been shown to cause an 80 to 90% reduction in the holding current of oocytes expressing $\alpha1\beta3\epsilon$ receptors (Neelands et al., 1999). The holding current of $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ receptors in the presence of 100 μM picrotoxin is similar to that observed in uninjected oocytes (S. A. Thompson and K. A. Wafford, unpublished observations), suggesting that 100 μM picrotoxin blocks the majority of the constitutively active channels. In addition the baseline (holding current) upon washout of tracazolate was not completely reestablished. A gradual reduction of the holding current was also observed for oocytes expressing $\alpha1\beta3\epsilon$ receptors, which were voltage-clamped at -70 mV and left for 1 to 2 h (data

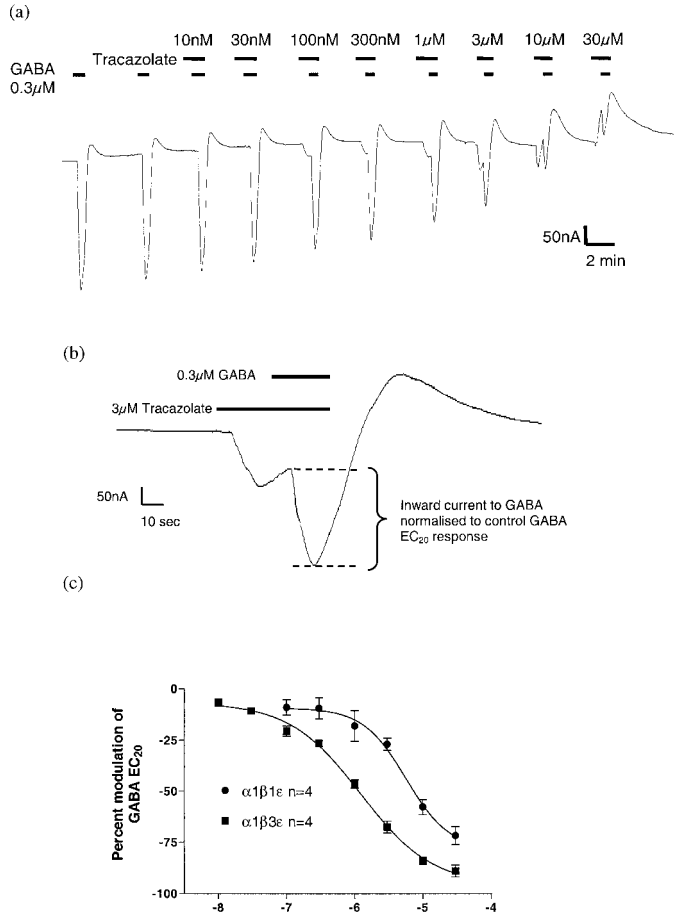


Fig. 4. Tracazolate inhibits control GABA EC_{20} responses on $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ receptors. A, representative trace showing a concentration-response curve to tracazolate on an oocyte expressing $\alpha1\beta3\epsilon$ receptors. The oocyte was voltage-clamped at -70 mV. The horizontal lines indicate application of tracazolate and GABA. Preapplication (30 s) of low concentrations of tracazolate (10 nM-1 μM) produced a small inward current, whereas concentrations above 1 μM produced an inward current followed by an outward current. B, expanded response to 3 μM tracazolate illustrating the inward current to GABA in the presence of tracazolate, which was normalized to the control GABA EC_{20} response. C, modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta1\epsilon$ (●) and $\alpha1\beta3\epsilon$ (■) $GABA_A$ receptors. Data represent the mean ± S.E.M. from the number of cells indicated from two or more batches of oocytes.

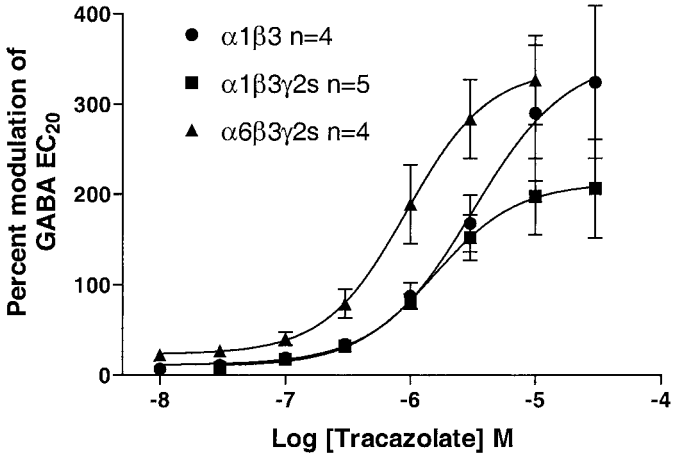


Fig. 3. Tracazolate potentiates control GABA EC_{20} responses on $\alpha1\beta3$ and $\alpha6\beta3\gamma2s$; interaction is not via the benzodiazepine binding site. Modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta3$ (●), $\alpha1\beta3\gamma2s$ (■), and $\alpha6\beta3\gamma2s$ (▲) $GABA_A$ receptors. Data represent the mean S.E.M. from the number of cells indicated from two or more batches of oocytes.

not shown), suggesting a long-term shift in the leak current possibly due to chloride redistribution through the constitutively active channel. Interestingly, the IC_{50} of the direct effect of tracazolate (i.e., inhibition of the constitutive activity) [1.4 (1.1, 1.6) μM, $n = 4$] was not significantly different from the IC_{50} value for inhibition of a GABA-activated EC_{20} response [1.2 (0.9, 1.5) μM, $n = 4$] (Fig. 5B).

The current-voltage relationship was determined for the direct effect of 3 μM tracazolate on $\alpha1\beta3\epsilon$ receptors. The data were best fitted to a linear regression ($r^2 = 0.94 \pm 0.02$, $n = 4$) and revealed a reversal potential of -25.7 ± 1.3 mV, $n = 4$, which was similar to the predicted reversal potential for Cl^- ions of -25.4 mV in *X. laevis* oocytes with an external Cl^- concentration of 89.91 mM (MBS used in this study) and an internal Cl^- concentration of 33.4 mM (Barish, 1983), indicating that the carrier of the direct effect is Cl^- ions.

Modulation of $\alpha1\beta1\gamma1$, $\alpha1\beta1\gamma3$, and $\alpha1\beta1\delta$ Receptors. The opposing effects observed with tracazolate on $\gamma2$s- and ϵ-containing receptors prompted studies on $\gamma1$-, $\gamma3$-, and δ-containing receptors. These subunits were coexpressed with $\alpha1$ and $\beta1$ subunits and concentration-response curves to tracazolate constructed. As can be seen in Fig. 6A tracazolate behaved

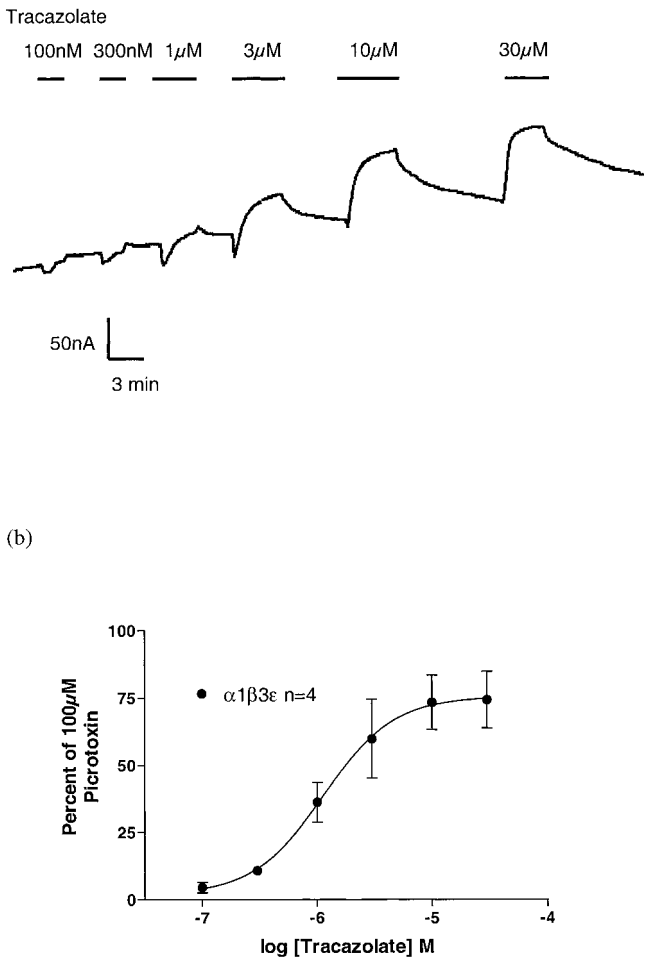


Fig. 5. Outward current to tracazolate on $\alpha1\beta3\epsilon$ receptors. A, representative trace showing the direct effect of tracazolate on an oocyte expressing $\alpha1\beta3\epsilon$ receptors. The oocyte was voltage-clamped at -70 mV. The horizontal lines indicate application of tracazolate. B, concentration-response curve for the outward current to tracazolate on $\alpha1\beta3\epsilon$ receptors (●) normalized to 100 μM picrotoxin. Data represent the mean ± S.E.M. from the number of cells indicated from two or more batches of oocytes.

TABLE 2

Summary of concentration-response data for tracazolate inhibition of control GABA EC_{20} responses for $\alpha1\beta1\epsilon$ and $\alpha1\beta3\epsilon$ $GABA_A$ receptors

Data for the EC_{50} are the geometric mean (−S.E.M., + S.E.M.) and for the maximum potentiation and Hill coefficient are the arithmetic mean ± S.E.M..

Subunit Combination	IC_{50}	Maximum Inhibition	Hill Coefficient	No. of Oocytes
	μM			
$\alpha1\beta1\epsilon$	4.0 (2.7, 5.9)	78.2 ± 9.3	−1.45 ± 9.3	4
$\alpha1\beta3\epsilon$	1.2 (0.9, 1.5)	96 ± 1.6	−0.84 ± 0.07	4

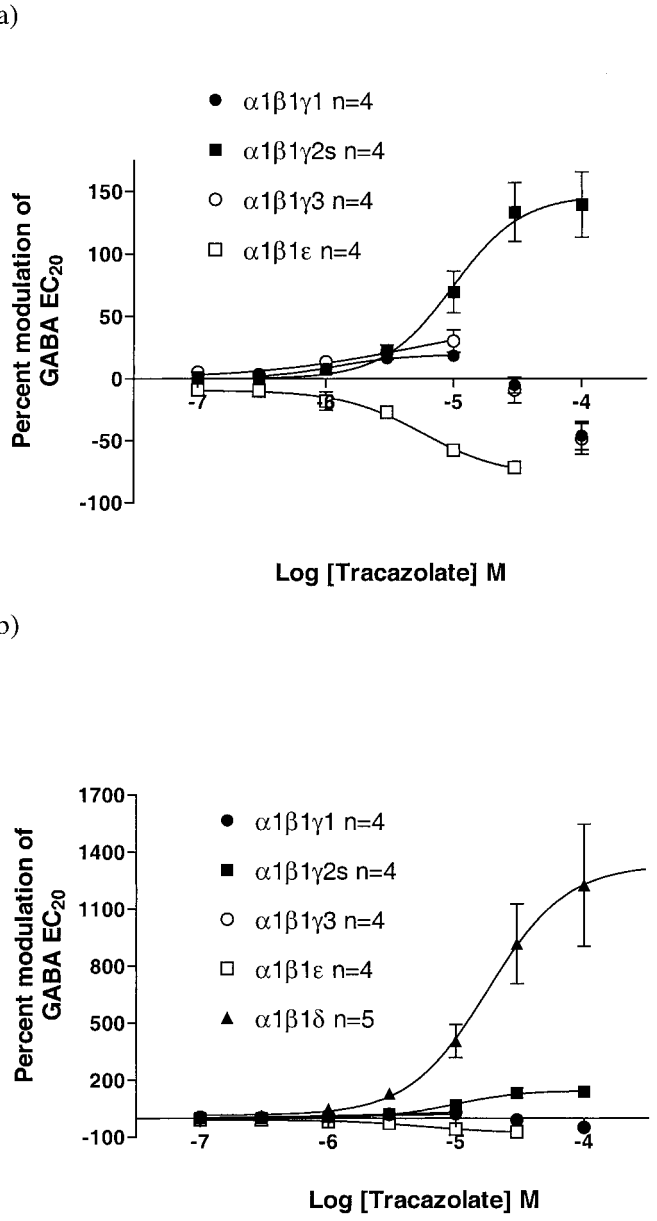


Fig. 6. Concentration-response curves to tracazolate on oocytes expressing $\alpha1\beta1\gamma1$, $\alpha1\beta1\gamma3$s, and $\alpha1\beta1\delta$ receptors. A, modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta1\gamma1$ (●), $\alpha1\beta1\gamma2$s (■), $\alpha1\beta1\gamma3$ (○), and $\alpha1\beta1\epsilon$ (□) $GABA_A$ receptors. B, modulation of the control GABA EC_{20} response by increasing concentrations of tracazolate on oocytes expressing $\alpha1\beta1\gamma1$ (●), $\alpha1\beta1\gamma2$s (■), $\alpha1\beta1\gamma3$ (○), $\alpha1\beta1\epsilon$ (□), and $\alpha1\beta1\delta$ (▲) $GABA_A$ receptors. Data represent the mean ± S.E.M. from the number of cells indicated from two or more batches of oocytes.

differently on $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$ receptors compared with $\alpha1\beta1\gamma2$s and $\alpha1\beta1\epsilon$. Concentrations up to 10 μM produced a small degree of potentiation of the GABA EC_{20} response (19 and 30% for 10 μM tracazolate on $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$, respectively), whereas higher concentrations inhibited the GABA EC_{20} response. The potentiating portion of the concentration-response curve revealed similar EC_{50} values, Hill coefficients, and maximum responses for $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$ receptors (Table 1). The data obtained for $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$ receptors were not significantly different from one another ($P > 0.05$, unpaired Student's t test); however, comparison with $\alpha1\beta1\gamma2$s revealed a 10-fold decrease in EC_{50}.

GABA EC_{20} responses for $\alpha1\beta1\delta$ receptors were potentiated by tracazolate to levels substantially greater than that produced by a maximum GABA concentration (Fig. 6B). Potentiation of a GABA EC_{20} concentration by tracazolate above and beyond the maximum current elicited by GABA was not observed with any other subunit combination examined. The log EC_{50} values and Hill coefficients, however, were not significantly different between $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2$s.

Effect of Tracazolate on GABA Concentration-Response Curves. The studies mentioned above only examined the effect that various concentrations of tracazolate have on a single, low concentration of GABA (EC_{20}). To further understand the mechanism of action of tracazolate, its effect on a range of GABA concentrations was examined. GABA concentration-response curves were constructed in the absence and then the presence of a single concentration of tracazolate on oocytes expressing $\alpha1\beta3\epsilon$, $\alpha1\beta3\gamma2$s, and $\alpha1\beta1\delta$ receptors.

On $\alpha1\beta3\gamma2$s receptors, 1 μM tracazolate produced a significant ($P < 0.05$) 2.5 $\pm$ 0.3-fold shift to the left of the GABA EC_{50} with no significant effect on the maximum response or Hill coefficient (Fig. 7A). Higher concentrations of tracazolate (10 and 30 μM) further increased this leftward shift of the GABA concentration-response curve (21.6 $\pm$ 5.5- and 39.3 $\pm$ 15.8-fold, respectively). In addition the maximum response of GABA in the presence of 10 and 30 μM tracazolate was significantly reduced compared with the maximum obtained for the control GABA concentration-response curve (67.6 $\pm$ 4 and 40.0 $\pm$ 2.9%, respectively). Tracazolate (30 μM) also significantly reduced the Hill coefficient of the GABA concentration-response curve compared with the control (1.54 $\pm$ 0.03 versus 0.98 $\pm$ 0.14, $P < 0.05$).

For $\alpha1\beta3\epsilon$ receptors, the inward current alone to GABA and tracazolate was measured omitting any direct effect. The log EC_{50} values and Hill coefficients for the control GABA concentration-response curves compared with those in the presence of 1 and 3 μM tracazolate were not significantly different, whereas the maximum response obtainable to GABA in the presence of 1 and 3 μM tracazolate were significantly lower ($P < 0.05$) (Fig. 7B).

Similar to $\alpha1\beta3\gamma2$s receptors, concentration-response curves to GABA on $\alpha1\beta1\delta$ receptors were shifted to the left by 10 and 30 μM tracazolate (7.6 $\pm$ 1.8- and 15.8 $\pm$ 1.9-fold, respectively). However, unlike $\alpha1\beta1\gamma2$s the maximum response to GABA in the presence of 10 and 30 μM tracazolate was significantly larger (202.9 $\pm$ 27.7 and 305.1 $\pm$ 34.2%, respectively) (Fig. 7C).

Domain 206 to 230 Determines Functional Response to Tracazolate. As described above, tracazolate is a positive modulator at receptors containing a γ subunit but a negative modulator when substituted by ϵ. To investigate the amino acid determinants of this effect, a series of chimeric $\gamma2/\epsilon$ subunits were constructed, each having an N-terminal $\gamma2$ domain. The junction between these two subunits was moved incrementally through to the start of TM2 from chimera C-A to C-D. The results from only the five most informative constructs are described previously (Fig. 8A). Chimeric subunits were coexpressed with $\alpha1\beta3$ and tested for modulation of a GABA EC_{20} response by 10 μM tracazolate. Tracazolate (10

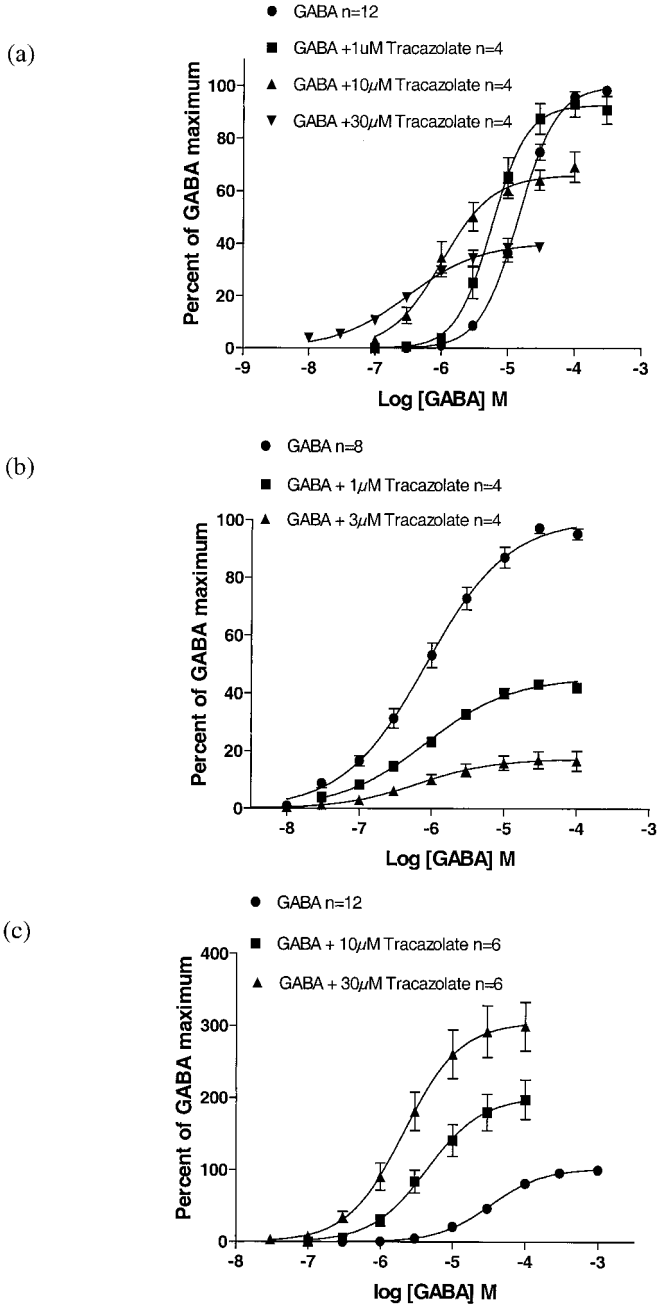
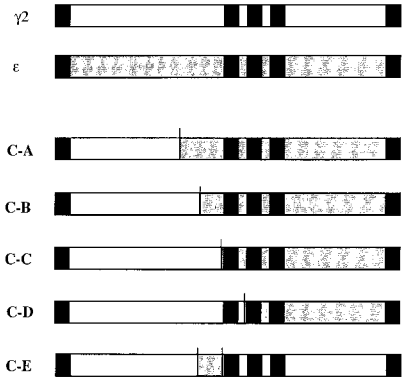


Fig. 7. Concentration-response curves for GABA in the absence and presence of tracazolate on $\alpha1\beta3\gamma2$s, $\alpha1\beta3\epsilon$, and $\alpha1\beta1\delta$. A, concentration-response curves for GABA (●), GABA + 1 μM tracazolate (■), GABA + 10 μM tracazolate (▲), and GABA + 30 μM tracazolate (▼) on $\alpha1\beta3\gamma2$s receptors. B, concentration-response curves for GABA (●), GABA + 1 μM tracazolate (■), and GABA + 3 μM tracazolate (▲) on $\alpha1\beta3\epsilon$ receptors. C, concentration-response curves for GABA (●), GABA + 10 μM tracazolate (■), and GABA + 30 μM tracazolate (▲) on $\alpha1\beta1\delta$ receptors. Data represent the mean $\pm$ S.E.M. from the number of cells indicated from two or more batches of oocytes.

μM) was chosen because this concentration produced the greatest window of separation between the potentiation on $\alpha1\beta3\gamma2s$ receptors and inhibition on $\alpha1\beta3\epsilon$ receptors. Similar to $\alpha1\beta3\epsilon$, chimera C-A and C-B were negatively modulated by tracazolate ($\alpha1\beta3\epsilon$: $-79.1 \pm 3.4\%$, $n = 6$; $\alpha1\beta3$C-A: $-46.4 \pm 8.1\%$, $n = 4$; and $\alpha1\beta3$C-B: $-80.4 \pm 6.6\%$, $n = 4$). Conversely, chimeras C-C and C-D were positively modulated by tracazolate to levels not significantly different from $\alpha1\beta3\gamma2s$ ($\alpha1\beta3$C-C: $113 \pm 15\%$, $n = 4$; $\alpha1\beta3$C-D: $88 \pm 6\%$, $n = 3$; and $\alpha1\beta3\gamma2s$: $198 \pm 42\%$, $n = 5$; Fig. 9). These results implicate a residue or residues within the $\gamma2$ domain 206 to 230 that confer the functional response observed with tracazolate. However, replacement of this whole region in $\gamma2$ with the homologous portion of ϵ (chimera C-E) did not alter the functional response to tracazolate [i.e., positive modulation similar to that of $\alpha1\beta3\gamma2$ receptors ($\alpha1\beta3$C-E: $210 \pm 51\%$, $n = 4$; Fig. 9)], suggesting that although this region may be necessary, other residues are also required to confer inhibition.

Discussion

Although tracazolate was first synthesized nearly 30 years ago, this is the first detailed electrophysiological study of its effects on recombinant $GABA_A$ receptors. Tracazolate binds to neither the benzodiazepine nor the GABA binding site but to another as-yet-unidentified site. Its potency (EC_{50}) is influenced by the nature of the β subunit; more importantly, however, its intrinsic efficacy (i.e., whether it potentiates or inhibits GABA) is critically determined by the nature of the third subunit ($\gamma1$–3, δ, or ϵ) within the receptor complex.



(b)

TM1

195 SWRLYQFSFVGlrNtTEvvkTtsGDyvVMsvyFdlSRRMGYFTIQTYIPCTLIVVLSW 252 γ2
221 SWKLFQFDFTGvsNkTEiitTpvGDfmVMtifFnvSRRFGYVAFQNYVPSSVTTMLSW 278 ε

Fig. 8. A, diagrammatic representation of $\gamma2/\epsilon$ chimeric subunits. The subunits are represented as rectangles with the putative signal peptide and transmembrane domains shown as black boxes. The $\gamma2$ subunit and portions created from it are in white and similarly the ϵ subunit is in gray. The five most informative chimeric subunits of those synthesized, C-A to C-E, are illustrated. The $\gamma2$ portion of each chimeric subunit is as follows: C-A, 1 to 147; C-B, 1 to 205; C-C, 1 to 230; C-D, 1 to 265; and C-E, 1 to 205 and 231 to 428 (mature peptide numbering). B, partial amino acid sequence alignment of the N termini of $\gamma2$ and ϵ subunits. The aligned amino acid sequences of the $\gamma2$ and ϵ subunits is shown for the region immediately N-terminal to, and including, the TM1, which is overlined. The domain identified as important for tracazolate function ($\gamma2$ residues 206–230) is boxed and within this region the nonconserved amino acids are in lowercase.

Tracazolate may prove to be a useful tool to aid identification of receptor subtypes within neuronal preparations.

Tracazolate Does Not Act via Benzodiazepine Binding Site. Tracazolate produced concentration-related potentiation of control GABA EC_{20} responses on oocytes expressing the binary receptor $\alpha1\beta3$. This result is in contrast to benzodiazepine compounds, which, at relevant concentrations, do not modulate $\alpha\beta$ receptors (Levitan et al., 1988; Pritchett et al., 1988). In addition, tracazolate did not displace [^{3}H]flumazenil from $\alpha3\beta3\gamma2s$ $GABA_A$ receptors stably expressed in Ltk^- cells (data not shown) nor was the functional response on $\alpha1\beta3\gamma2s$ receptors inhibited by flumazenil.

The type of β subunit present within the receptor complex has previously been shown not to effect the modulation obtained with benzodiazepine site ligands (Hadingham et al., 1993). Tracazolate, however, was significantly more potent on $\alpha1\beta3\gamma2s$ receptors compared with $\alpha1\beta1\gamma2s$ receptors. Recently, an increasing number of structurally unrelated compounds have been identified that also show this selectivity (e.g., loreclezole, β-carbolines, etomidate, furosemide, and mefenamic acid). For each compound, this selectivity has been shown to be due to the presence of an asparagine residue at the homologous position 264 and 265 within TM2 of the $\beta2$ and $\beta3$ subunit, respectively. Similarly, this asparagine residue was shown to be responsible for the $\beta3$ selectivity observed with tracazolate. Collectively, these results demonstrate that tracazolate does not interact with the benzodiazepine site and are in agreement with the previous biochemical and electrophysiological data obtained for the pyrazolopyridines (Williams and Risley, 1979; Barnes et al., 1983).

Importance of Third Subunit within Receptor Complex. The nature of the third subunit within the receptor complex was critical in determining the functional response to tracazolate. For $\alpha1\beta1/3\gamma2s$ receptors tracazolate produced concentration-related potentiation of control GABA EC_{20} responses; however, for $\alpha1\beta1/3\epsilon$ receptors GABA EC_{20} responses were inhibited by tracazolate. Receptors containing a $\gamma1$ or a $\gamma3$ subunit produced an intermediate profile with low concentrations of tracazolate potentiating to a small degree the GABA EC_{20}, whereas higher concentrations caused inhibition. These differing functional effects are in contrast to the general anesthetic agents such as pentobarbitone and propofol and the neuroactive steroids, which potentiate all the receptor subtypes examined herein (Whiting et al., 1997; Thompson et al., 1998; Maitra and Reynolds, 1999). In addition, these agents are also dissimilar to tracazolate because they do not display β subunit selectivity.

The largest degree of potentiation was observed with

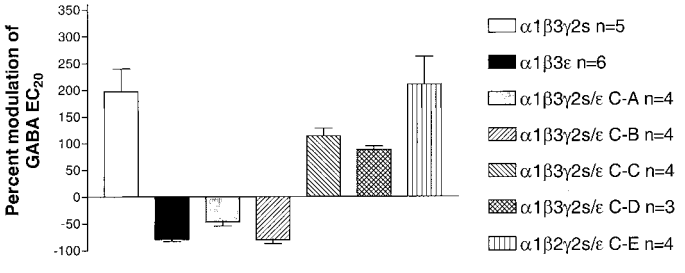


Fig. 9. Tracazolate modulation of receptors containing chimeric $\gamma2s/\epsilon$ subunits. Wild-type and chimeric subunits were coexpressed with $\alpha1\beta3$ in *X. laevis* oocytes and assayed for modulation of a GABA EC_{20} response by 10 μM tracazolate. Data represent the mean $\pm$ S.E.M. from the number of cells indicated from two or more batches of oocytes.

$\alpha1\beta1\delta$ receptors. On this receptor subtype, tracazolate potentiated the GABA EC_{20} response by 1368 ± 377%. This current was approximately 3 times that elicited by a maximum concentration of GABA. Potentiation of a GABA $EC_{10–25}$ response beyond that of the maximum GABA response has previously been demonstrated for isoflurane on $\alpha1\beta1\delta$ receptors (Lees and Edwards, 1998). One possible explanation of these results is that on $\alpha1\beta1\delta$ receptors GABA behaves as a partial agonist with a low probability of opening. This probability of opening is increased in the presence of tracazolate or isoflurane, giving rise to a supramaximal response. Further evidence for GABA behaving as a partial agonist has been demonstrated using an Ltk^- cell line stably expressing $\alpha4\beta3\delta$ receptors in which concentration-response curves to 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol elicit significantly larger responses than GABA (Adkins et al., 2001; Brown et al., 2001).

Outward Current to Tracazolate on $\alpha1\beta3\epsilon$ Receptors. Spontaneous channel openings in the absence of GABA have been demonstrated in outside-out patches pulled from fibroblasts transiently transfected with $\alpha1\beta3\epsilon$ (Neelands et al., 1999). In addition to picrotoxin and tracazolate, Zn^{2+} ions and bicuculline have been shown to elicit outward currents on $\alpha1\beta3\epsilon$ receptors (Neelands et al., 1999). One explanation for the outward current observed with these agents is that they inhibit the constitutively active currents. Mutation of the 9′ leucine residue within the $\beta2$ subunit and coexpression with $\alpha1\gamma2s$ produced a receptor with a high degree of constitutive activity, which, like $\alpha1\beta3\epsilon$ receptors, was reduced in the presence of picrotoxin, bicuculline, gabazine, and Zn^{2+} (Thompson et al., 1999b). Our results demonstrate that the outward current to tracazolate on $\alpha1\beta3\epsilon$ receptors is carried by chloride ions, suggesting that like other agents it inhibits constitutive activity. The small inward current to low concentrations of tracazolate may represent initial potentiation of the constitutive activity, which is then superseded by the outward current.

Differing Effects on GABA Concentration-Response Curves. Concentration-response curves to GABA in the absence and presence of tracazolate on $\alpha1\beta3\gamma2s$, $\alpha1\beta3\epsilon$, and $\alpha1\beta1\delta$ receptors revealed further insights into the mechanism of action of tracazolate. GABA concentration-response curves on both $\alpha1\beta3\gamma2s$ and $\alpha1\beta1\delta$ receptors were shifted to the left with significantly lower EC_{50} values. The maximum response to GABA, however, in the presence of increasing concentrations of tracazolate, were shifted in opposing directions; on $\alpha1\beta3\gamma2s$ tracazolate reduced the maximum response to GABA, whereas on $\alpha1\beta1\delta$ this was increased. The leftward shift in the GABA concentration-response curve with a reduction in the maximum response for $\alpha1\beta3\gamma2s$ receptors is similar to that reported for loreclezole (Wafford et al., 1994) and SB-205384 (Meadows et al., 1997) and may indicate a common mechanism of action of these compounds. The similarities of these three compounds also extend to the selectivity for $\beta2/3$-containing receptors over $\beta1$-containing receptors. Benzodiazepine site ligands also produce a leftward shift in the GABA concentration-response curve; however, they cause no reduction in the maximum response (Sigel and Baur, 1988; Maksay et al., 2000).

On $\alpha1\beta3\epsilon$ receptors tracazolate behaved as a noncompetitive antagonist, reducing the maximum response to GABA with no change in the log EC_{50} value or Hill coefficients.

Identification of Region Critical for Functional Efficacy of Tracazolate. Chimeras C-A and C-B when coexpressed with $\alpha1\beta3$ subunits revealed similar characteristics to $\alpha1\beta3\epsilon$ receptors (i.e., inhibition of the GABA EC_{20} response by tracazolate), a direct effect to tracazolate, and the presence of a rebound current upon washout of GABA. Chimeras C-C and C-D, however, were similar to $\alpha1\beta3\gamma2s$ receptors; i.e., they were potentiated by tracazolate and showed no direct effect to tracazolate or rebound currents upon washout of GABA. The switch from negative to positive modulation occurred between chimera C-B to C-C, implicating the region equivalent to $\gamma2$ residues 206 to 230 in determining the direction of modulation by tracazolate. However, the potentiation observed with chimera C-E, in which only this domain of $\gamma2$ was replaced by that of ϵ, suggests a role for an additional C-terminal element in the transduction process, indicating that this domain is necessary but not sufficient to confer ϵ-like inhibition.

This region just before TM1 is in the vicinity of the loop C domain, which includes several amino acid positions that influence ligand binding in subunits of the Cys-loop receptor family (Vafa and Schofield, 1998). The loop C ligand-binding domain of the α subunit has several amino acid positions that have been suggested to have a role in the function of benzodiazepine site ligands [e.g., positions 201 (Pritchett and Seeburg, 1991), 205 (Renard et al., 1999), and 207 and 210 (Amin et al., 1997; Buhr et al., 1997)]. Boileau et al. (1998) and Boileau and Czajkowski (1999) have investigated the mechanism of benzodiazepine action using chimeric $\gamma2/\alpha1$ subunits. These studies are not easily comparable with those carried out herein because the chimeras used were constructed from subunits of nonequivalent classes. Nevertheless, it is interesting to note that the region of the subunit that was identified as being important for benzodiazepine potentiation is overlapping with that which we have identified in this study. From these data, it is clear that the region just before TM1 is important for allosteric effects, perhaps not surprising given its vicinity to the channel.

Mechanism of Action. The results obtained in this study lead us to speculate on the possible mechanism of action of tracazolate. We hypothesize that a single common site is present, and the observed inhibition or potentiation relates to the nature of the GABA subtype. This is supported by the same apparent EC_{50} for inhibition or potentiation and an identical shift of EC_{50}/IC_{50} when the β subunit is switched. We observed that under conditions where the receptor rapidly entered the desensitized state (e.g., $\alpha1\beta1/3\epsilon$, or high concentrations of GABA on $\alpha1\beta3\gamma2s$) the functional response to tracazolate was inhibition, whereas in conditions with little desensitization (e.g., $\alpha1\beta1\delta$ or low GABA concentrations on $\alpha1\beta3\gamma2s$) the functional response was potentiation. The $GABA_A$ receptor subtypes compared in this study differ markedly in their rate of desensitization, with $\alpha\beta\epsilon$ receptors desensitizing faster than $\alpha\beta\gamma2s$, which in turn desensitize faster than $\alpha\beta\delta$ receptors (Saxena and Macdonald, 1994; Whiting et al., 1997; Brown et al., 2001). One possible interpretation of the data is that tracazolate displays higher affinity for the desensitized state than for the agonist bound state of the receptor. A similar mechanism has been proposed for the action of ifenprodil on *N*-methyl-D-aspartate receptors (Kew et al., 1996). Experiments to investigate the effects of tracazolate on the kinetics of GABA will be required to validate this hypothesis.

In conclusion, this study represents the first functional characterization of the modulatory effects of tracazolate on recombinant $GABA_A$ receptors and reveals that tracazolate has a unique profile unlike any other allosteric modulator characterized to date. Mutagenesis studies, aimed at identifying the molecular determinants responsible for the opposing functional effects of tracazolate on γ- and ϵ-containing receptors have highlighted region 206 to 230 in γ2 as being necessary but not sufficient in determining the different functional effects.

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