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Subunit-Specific Action of an Anticonvulsant Thiobutyrolactone on Recombinant Glycine Receptors Involves a Residue in the M2 Membrane-Spanning Region

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ABSTRACT

The anticonvulsant α-ethyl, α-methyl-γ-thiobutyrolactone (αEMTBL) potentiates the response to a submaximal concentration of glycine produced by receptors composed of human glycine α1-subunits but reduces the response of receptors composed of rat glycine α3-subunits. Both the potentiating and blocking actions of αEMTBL are reduced by higher concentrations of glycine. The subunit specificity of αEMTBL block is conferred by a residue in the second membrane-spanning region (M2), which is alanine in the α3-subunit (A254) and glycine in the α1-subunit. The mutation A254G in the α3-subunit removes blocking by αEMTBL and reveals potentiation. Picrotin, a picrotoxinin analog, blocks responses of receptors composed of either α1 or α3-subunits. Blocking of α3 receptors by picrotin is reduced in the presence of αEMTBL, indicating that the mechanisms interact at some point, but the mutation α3 A254G does not remove block by picrotin. However, mutation of a nearby residue α3 T258F does remove block by picrotin, picrotoxinin and αEMTBL. These observations suggest that αEMTBL and picrotin act on glycine α3 receptors to produce block by an allosteric mechanism that involves overlapping sets of residues in the M2 region. Coexpression of the α3-subunit with the β-subunit of the glycine receptor also removes block by αEMTBL and reveals potentiation, suggesting that receptors containing either α3 or α1 glycine receptor subunits are potentiated in the adult brain.

γ-Butyrolactones and structurally related drugs can act as convulsants or anticonvulsants. A series of studies of the mechanism of action of these drugs has indicated that a major site of action is on the γ-aminobutyric acid A ($GABA_A$) receptor (Holland et al., 1990). The anticonvulsant drugs potentiate the activation of the $GABA_A$ receptor, whereas the convulsant drugs block the receptor. It was initially thought that both classes of drugs acted by binding to the same site on the $GABA_A$ receptor as picrotoxinin; the convulsant drugs as agonists (mimicking the action of picrotoxinin) and anticonvulsant drugs as inverse agonists (Holland et al., 1990). Subsequent work has demonstrated that this hypothesis is not accurate (Holland et al., 1993). Instead, potentiation (anticonvulsant activity) is mediated after binding to an unidentified site that differs from the GABA-binding site, the barbiturate-binding site, and the benzodiazepine-binding site (Holland et al., 1993, 1995). However, receptor block may result from interactions with the picrotoxin binding site (Yoon et al., 1993; Xu et al., 1995; Williams et al., 1997).

We have undertaken studies of a related receptor, the glycine receptor, to examine the molecular mechanism of lactone actions. The studies are facilitated because functional glycine receptors can be expressed as homomultimers of a single subunit. We found that receptors composed of glycine α1-subunits are potentiated by an anticonvulsant thiobutyrolactone, α-ethyl-α-methyl-thiobutyrolactone (αEMTBL), in a fashion similar to that of $GABA_A$ receptors. Surprisingly, however, receptors composed of glycine α3-subunits are blocked by similar concentrations of αEMTBL. We pursued this observation to examine the mechanism by which αEMTBL blocks responses of the α3 receptor and to determine the structural basis for the subunit specificity. In addition, we made comparative studies of the blocking action of the drug picrotin, which is structurally related to picrotoxinin and has already been shown to block glycine α1 receptors (Lynch et al., 1995). The observations show that both αEMTBL and picrotin block responses in a competitive manner (which is not consistent with an open-channel-blocking mechanism), and that both mechanisms of block are affected by residues in the postulated channel-lining portion of the receptor. The observations are consistent with the idea that

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ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid A; αEMTBL, α-ethyl, α-methyl-γ-thiobutyrolactone; βEMGBL, β-ethyl-β-methyl-γ-butyrolactone; CTB, cyanotriphenyl borate; DMSO, dimethyl sulfoxide.

αEMTBL and picrotin block responses by an allosteric action on the glycine receptor.

In the adult rat brain, most glycine receptors are heteromultimers composed of α1 and β-subunits, although in some regions the α3 and β-subunits are expressed (Vannier and Triller, 1997). We found that coexpression of α1 and β-subunits had no effect on potentiation, whereas coexpression of α3 and β-subunits removed block. This observation suggests that, in the adult brain, glycine receptors containing either α1 or α3-subunits would be potentiated by αEMTBL.

Materials and Methods

αEMTBL was synthesized as described (Levine et al., 1986). Other drugs were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Constructs for glycine receptor rat β (Grenningloh et al., 1990a) and rat α3 (Kuhse et al., 1990) subunits were kindly provided by Dr. H. Betz (Max-Planck-Institut fur Hirnforschung, Frankfurt) in the pCIS2 vector. The human α1-subunit (Grenningloh et al., 1990b) was kindly provided by Dr. P. Schofield (University of New South Wales, Sydney) also in the pCIS2 vector. Two pairs of reciprocal chimeras were produced by joining the α1 and α3-subunits at the following amino acid residues (all residues are numbered for the mature subunit): c1 α1(216)/α3(217), c1 α3(216)/α1(217), c2 α1(340)/α3(356), c2 α3(355)/α1(341). To do this, an *Xba*I site was mutated into the α3-subunit (c1) and an in-frame *Apa*I site was mutated into the α1-subunit (c2), using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were introduced into the α3-subunit, also using QuikChange. A portion of the mutated subunit was excised and transferred to wild type subunits, and the transferred region was sequenced completely to confirm that the appropriate mutation was produced, and no additional mutations had been introduced.

Glycine receptors were expressed in *Xenopus* oocytes by injecting cDNA into the nucleus, using the blind method described by Colman (1984). Nuclei were injected with 13.6 nl of solution containing cDNA at about 1 μg/ml. When α- and β-subunits were coexpressed, the β cDNA was present at a 5-fold higher concentration. Responses were recorded 2 to 5 days after injecting the oocytes, at a holding potential of −50 mV using a two-electrode oocyte clamp (Warner Instruments, New Haven, CT). Both voltage and current electrodes were patch-clamp electrodes filled with 3 M KCl, and had resistances of 0.5 to 1 MOhm. The bath solution contained (mM) NaCl, 96; KCl, 2; $MgCl_2$, 1; $CaCl_2$, 1.8; HEPES, 10; pH was adjusted to 7.5 with addition of NaOH. The bath had a volume of about 0.1 ml, and was perfused with saline or drug solutions at about 7 ml/min. Solutions were switched by hand, using Teflon rotary valves (Rheodyne, Rohnert Park, CA). To avoid loss of hydrophobic compounds, glass perfusion reservoirs were used and all tubing was Teflon or metal. Drugs were dissolved in bath solution. αEMTBL was added to solutions containing 0.2% (v/v) dimethyl sulfoxide (DMSO). To dissolve αEMTBL at 10 mM, the solution was heated to 70°C for about 30 min, and mixed extensively. Higher concentrations of αEMTBL were not tested, due to limited aqueous solubility. Applications lasted 5 to 20 s, until the response had reached a plateau, and were separated by 60 s (at low concentrations) to 240 s (at high concentrations). We noted that the response amplitude often changed slowly over time, so in all cases a standard control concentration of glycine was applied bracketing the test applications. The usual control concentration was 100 μM glycine, whereas the control for applications of αEMTBL was 100 μM glycine plus 0.2% DMSO.

Data are presented as the response normalized to the mean of the bracketing control responses. Data values are presented in the text and shown in the figures as mean ± S.E. (*N* cells). The significance of differences was assessed using a two-tailed Student's *t* test for unpaired observations, assuming unequal variances.

Results

Xenopus oocytes injected with cDNA coding for the glycine human α1-subunit or rat α3-subunit responded to applications of glycine. The concentration-response curves (Fig. 1) could be described by the Hill equation, with values for the concentration producing half-maximal activation (EC_{50}) and the Hill coefficient (*n*) of: α1 EC_{50} = 212 ± 28 μM and *n* = 2.0 ± 0.2 (mean ± S.E., 12 cells), α3 EC_{50} = 531 ± 84 μM and *n* = 2.3 ± 0.4 (6 cells). These values are similar to those reported in earlier studies of glycine receptors expressed in *Xenopus* oocytes [compare with Kuhse et al. (1990) and Taleb and Betz (1994)].

αEMTBL Potentiates Responses of α1 Homomultimeric Receptors and Blocks Those of α3. αEMTBL potentiated the response of α1 glycine receptors to 100 μM glycine (Fig. 2), in a similar fashion to its actions on $GABA_A$ receptors. However, αEMTBL blocked the response of α3 glycine receptors over the same concentration range (Fig. 2). The block produced by αEMTBL was not complete (Fig. 2). Indeed, the fact that block by 10 mM αEMTBL was no greater than by 3 mM suggests that even for α3 receptors some potentiation might be present.

Because the ability of many drugs to act on receptors is affected by the concentration of agonist used to activate the receptor, we tested the effect of 3 mM αEMTBL on the responses to a range of glycine concentrations (Fig. 3A). The block of responses from α3 receptors was reduced at high glycine concentrations (for glycine concentrations of 1 or 10 mM, the response in the presence of 3 mM αEMTBL did not differ from that in its absence; $P > .2$). The block of responses from α3 receptors by αEMTBL, therefore, has some features of a competitive block. A simple block of open channels would not behave in this fashion. If open channels were preferentially blocked by αEMTBL the fractional block by a constant

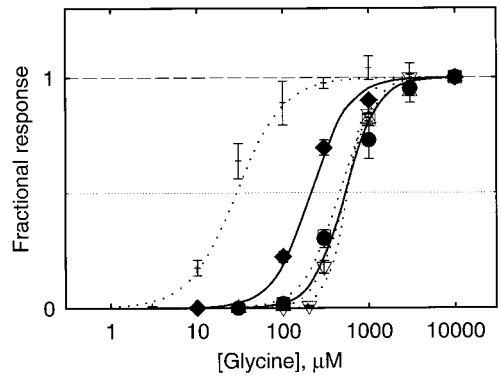

Fig. 1. Concentration-response relationship for activation by glycine. This figure shows the normalized response elicited by a given concentration of glycine, plotted against the glycine concentration. Filled symbols show data for α1 receptors (diamonds) and α3 receptors (circles), whereas solid lines show predictions of the Hill equation with the average parameters given below. Open symbols show results obtained with the mutated subunits α3 V240I (squares; obscured under the α3 points), α3 A254G (upright triangles), α3 V240I/A254G (inverted triangles), and α3 T258F (crosses), whereas dotted lines show predictions of the Hill equation for each construct. Data are shown as mean ± S.E. (when larger than the size of the symbol). When the Hill equation was used to describe the data, the values for the Hill coefficient and EC_{50} (given as mean ± S.E., number of activation curves analyzed) were: α3: 2.28 ± 0.48, 531 ± 84 μM (6); α3 V240I: 2.06 ± 0.22, 457 ± 14 μM (3); α3 A254G: 2.63 ± 0.11, 531 ± 39 μM (3); α3 V240I/A254G: 3.79 ± 0.78, 558 ± 62 μM (8); α1: 1.95 ± 0.16, 212 ± 28 μM (12); α3 T258F: 1.58 ± 0.28, 29 ± 8 μM (5), respectively.

concentration of αEMTBL would be expected to increase at higher concentrations of glycine.

The block produced by αEMTBL did not depend on membrane potential. The ability of 3 mM αEMTBL to block responses to 100 μM glycine was determine at −100, −50, and 0 mV in four oocytes. The mean relative currents in the presence of αEMTBL were 0.36 ± 0.05, 0.34 ± 0.05, and 0.30 ± 0.05, respectively. These amounts of block did not differ significantly at different potentials ($P > .4$ for comparisons between potentials, paired t test). Actually, because αEMTBL is uncharged, it is perhaps not surprising that no significant dependence of block on membrane potential was observed.

Similarly, potentiation of responses from α1 receptors was reduced at high glycine responses (for glycine concentrations of 1, 3, or 10 mM, the response in the presence of 3 mM αEMTBL did not differ from that in the absence; $P > .15$). In other words, αEMTBL did not increase the maximal response of α1 receptors to glycine. A similar observation has been made for potentiation of responses from $GABA_A$ receptors (Hill et al., 1998).

Although the extent of block or potentiation by αEMTBL depended on the concentration of glycine used to elicit the response, the qualitative nature of the effect (block or potentiation) did not. The quantitative actions of αEMTBL were greatest at lower concentrations of glycine, which activated a smaller fraction of the maximal current. The test concentrations of glycine used in the experiments for the various constructs are shown in Table 1, together with the fractional current activated by that concentration of glycine. In general, 100 μM glycine was used.

Block by Picrotoxinin and Picrotin. The actions of the blocking drugs picrotoxinin and picrotin on these receptors were also examined. We confirmed the report that picrotin is as effective at blocking responses of α1 receptors as is picrotoxinin (Lynch et al., 1995): 100 μM picrotoxinin reduced responses to 0.03 ± 0.006 of the control response (13 cells), whereas 100 μM picrotin reduced responses to 0.04 ± 0.005 (5 cells). We found similar results with α3 receptors: 100 μM picrotoxinin reduced responses to 0.14 ± 0.09 (4 cells),

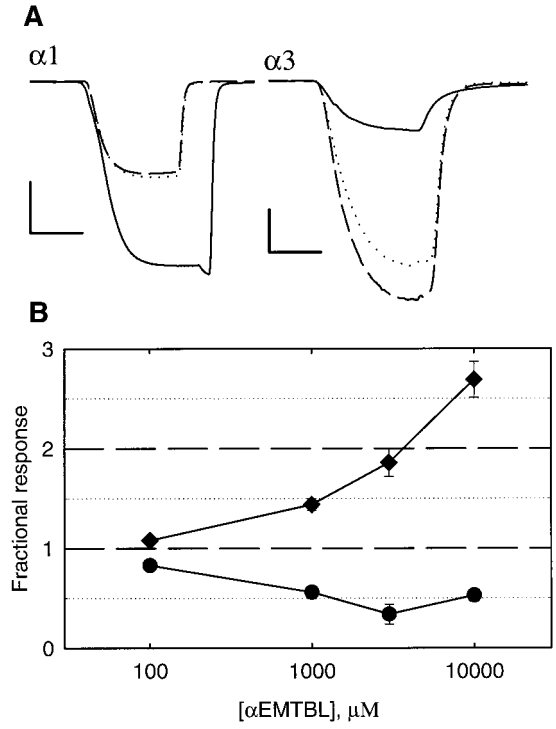

Fig. 2. αEMTBL potentiates responses of α1 receptors and blocks responses of α3 receptors. The upper part of the figure shows recordings of current from oocytes expressing α1 receptors (left) or α3 receptors (right). In each set, the solid trace shows the response to 100 μM glycine in the presence of 3 mM αEMTBL, whereas control responses are shown as dashed (preceding control) and dotted (recovery) traces. The scale bars are 200 nA, 10 s (α1, left) and 20 nA, 10 s (α3, right). The lower part of the figure shows the concentration effect curves for the action of αEMTBL, expressed as the fractional response in the presence of αEMTBL relative to the mean of the preceding and recovery responses to the test concentration of glycine in the absence of αEMTBL. Potentiation results in a fractional response >1, whereas block produces a fractional response <1. Solid symbols show actions on α1 receptors (diamonds) and α3 receptors (circles); the lines simply connect the points. Data are shown as mean ± S.E. (when larger than the size of the symbol) for data from 3 to 11 cells.

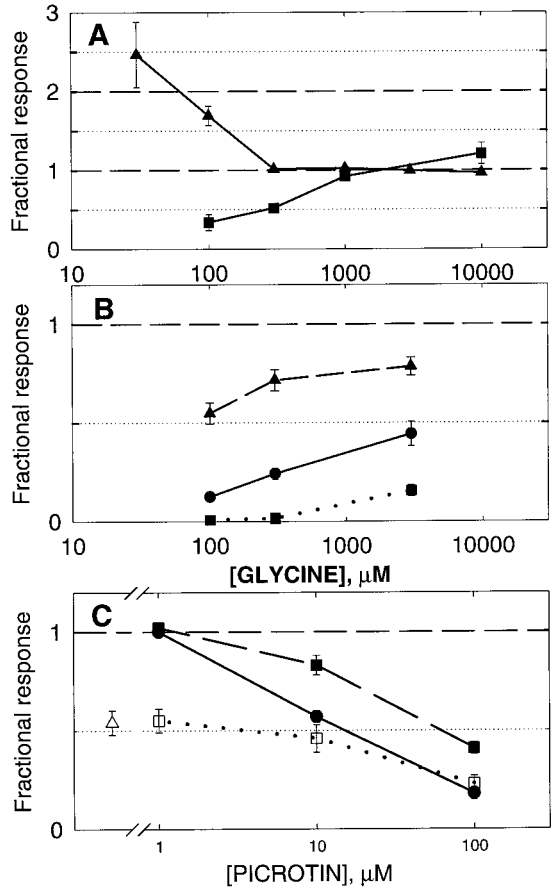

Fig. 3. Interactions between αEMTBL, picrotin, and glycine. A, the ability of 3 mM αEMTBL to potentiate responses of α1 receptors (triangles) and to block responses of α3 receptors (squares) is reduced at high glycine concentrations. B, the ability of picrotin (10 μM, triangles; 100 μM, circles; and 1 mM, squares) to block responses of α3 receptors is also reduced at high glycine concentrations. Data show mean ± S.E., for data from 3 to 11 oocytes. C, results of an experiment which tested for interaction between picrotin and αEMTBL. Four oocytes were tested using 300 μM glycine to elicit responses. Block produced by picrotin alone (filled circles and solid line), by 3 mM αEMTBL alone (open triangle), and by picrotin in the presence of 3 mM αEMTBL (open squares and dotted line) was then measured. The response in the presence of both αEMTBL and picrotin was renormalized to the response of the same oocyte in the presence of αEMTBL alone (filled squares and dashed line). A paired t test on the fractional block showed that picrotin produced less block of the residual response in the presence of αEMTBL than of the response in the absence of αEMTBL (100 μM picrotin, $P < .001$; 10 μM picrotin, $P < .005$). Data show mean ± S.E., for data from four oocytes.

whereas 100 μM picrotin reduced responses to 0.10 ± 0.02 (9 cells). For each receptor type, the amounts of block produced by 100 μM picrotin and 100 μM picrotoxinin were statistically indistinguishable.

Block of responses from α3 receptors by picrotin was reduced at high concentrations of glycine (Fig. 3B), as already reported for the action of picrotoxinin on responses of α1 receptors (Lynch et al., 1995). This observation is similar to that made for αEMTBL.

Effect of Coexpression of the β-Subunit and Interactions between Picrotin and αEMTBL. Coexpression of the β-subunit with an α-subunit is known to greatly reduce the sensitivity of the glycine receptor to picrotoxinin (Pribilla et al., 1992), and also reduced the sensitivity of the receptor to picrotin (Table 1). In addition, coexpression of the β-subunit with the α3-subunit removed the ability of αEMTBL to block responses (Table 1), and revealed potentiation ($P < .02$ that the response in the presence of αEMTBL differs from control). This observation suggests similarities in the mechanism of action of αEMTBL and picrotin. Coexpression of the β-subunit with the α1-subunit did not remove potentiation of responses by αEMTBL (Table 1).

Finally, the ability of picrotin to block the residual response in the presence of αEMTBL was examined. If there were no interaction between the two drugs, it would be expected that the residual response would be blocked equivalently to the control response in the absence of αEMTBL. However, the presence of 3 mM αEMTBL altered the concentration dependence of the block by picrotin (Fig. 3C). The normalized blocking curve for picrotin in the presence of αEMTBL is clearly shifted to higher concentrations. These data suggest that the mechanisms of block by picrotin and αEMTBL converge at some level.

These observations show that block of α3 homomultimeric receptors by αEMTBL shares features with block by picrotin and suggest that the mechanisms may converge at some point. The differential effects of αEMTBL on α1 and α3 homomeric receptors clearly distinguish the two drugs, however.

Amino Acid Residues Determining the Subunit-Specific Actions of αEMTBL. To map the region involved in determining the ability of αEMTBL to produce block as opposed to potentiation, four chimeric subunits were constructed between the α1- and α3-subunits (Fig. 4A). The chimeras were designed to separate the subunits into three sections: the N-terminal external region, the M1 through M3 membrane-spanning region, and the cytoplasmic loop plus M4 membrane-spanning region. The N-terminal external region contains regions important for binding ligands, including glycine and strychnine. The M2 region contains residues forming a major portion of the ion channel, residues important for binding noncompetitive antagonists, and residues that significantly affect channel conformational changes. The cytoplasmic loop and M4 regions have been studied less extensively (reviewed in Rajendra et al., 1997). The chimeras that contained the membrane-spanning regions M1, M2, and M3 from the α3-subunit showed block by αEMTBL, whereas those with the same regions from the α1-subunit showed potentiation. There was no systematic dependence on the origin of the N-terminal extracellular loop or on the cytoplasmic loop and M4 region (Fig. 4A).

The α1- and α3-subunits are highly homologous between the two chimera-joining points. In fact, they differ at only two positions in the membrane-spanning regions (Fig. 4B), one in M1 (α1 I240, α3 V240) and the second in M2 (α1 G254, α3 A254). There are additional differences in the cytoplasmic loop between the end of M3 and the joining site at c2, but these differences appeared less likely to be important in the actions of αEMTBL.

The residues in M1 and M2 were mutated in the rat α3-subunit to the amino acid found in the α1 receptor, separately and together. As shown in Fig. 1, activation by glycine was essentially unaffected in either of the two single mutants. Accordingly, 100 μM glycine was used as the test concentration (activating about 2% of the maximal response, Table 1).

Mutation of the residue in M1 (α3 V240I) produced no significant effect on the action of αEMTBL, although there was a marginal increase in block.

Mutation of the residue in M2 (α3 A254G) removed the blocking action of αEMTBL (Table 1). Indeed, the response was potentiated in the presence of αEMTBL (the response in the presence of αEMTBL differed significantly from that in its absence, $P < .005$).

The double mutant subunit α3 A254G/V240I showed a change in activation by glycine, in that the Hill coefficient for activation was increased and the fractional activation by 100 μM glycine was reduced (Fig. 1). Accordingly, 200 μM glycine

TABLE 1

Actions of αEMTBL and picrotin on wild type and mutated receptors

The first column gives the subunit(s) expressed, while the second column gives the test concentration of glycine used. The third column shows the faction of the maximal response elicited by the test concentration of glycine. The fifth column shows the fractional response elicited by the test concentration when applied in the presence of 3 mM αEMTBL relative to the response in the absence of αEMTBL, whereas the seventh column shows similar data for 100 μM picrotin. The columns headed *P* give the results of two-tailed *t* tests comparing the fractional responses obtained for a given construct to the fractional responses from wild type α3 receptors (NS: $P > .05$).

Construct	[Gly]	Test Response	Response with αEMTBL	*P*	Response with Picrotin	*P*
	μM					
α3	100	0.02 ± 0.01 (8)	0.34 ± 0.10 (8)		0.10 ± 0.02 (9)	
α3 V240I	100	0.02 ± 0.01 (4)	0.15 ± 0.03 (6)	NS	0.13 ± 0.04 (5)	NS
α3 A254G	100	0.01 ± 0.004 (5)	4.67 ± 0.69 (6)	0.002	0.35 ± 0.02 (5)	<0.001
α3 V240I/A254G	200	0.01 ± 0.001 (4)	7.48 ± 1.28 (4)	0.01	0.20 ± 0.04 (7)	NS
α1	100	0.22 ± 0.02 (16)	1.86 ± 0.14 (11)	<0.001	0.04 ± 0.005 (5)	0.04
α3 T258F	10	0.17 ± 0.03 (10)	3.84 ± 1.89 (4)	—[a]	1.15 ± 0.15 (6)	0.001
α3β	100	0.02 ± 0.01 (7)	1.74 ± 0.24 (7)	0.001	1.07 ± 0.11 (15)	<0.001
α1β	100	0.24, 0.30	1.47, 1.23	0.008	0.75 ± 0.09 (5)	0.002

[a] αEMTBL potentiated responses from α3 T258F receptors (four of four oocytes tested), but the *t* test resulted in a nonsignificant probability for the difference to wild type α3. The Mann-Whitney test comparing data from α3 T258F to wild type α3 provided a *P* value of .02. Data are shown as mean ± S.E. (number of oocytes tested) or as individual values when fewer than four oocytes were studied.

was used as the test concentration (Table 1). This double mutant also showed potentiation by αEMTBL (Table 1; $P < .02$). The residue in M2, therefore, determines the difference in the actions of αEMTBL on these glycine receptors.

The ability of picrotin to block responses was not removed in any of the mutated receptors (Table 1). However, the block by 100 μM picrotin was significantly reduced in α3 A254G and to a lesser extent in α3 A254G/V240I (Table 1). This observation will require additional experimentation to fully resolve, because the blocking efficacy depends on both the activation by glycine and block by picrotin (see above).

An additional mutated α3-subunit was generated, α3 T258F, based on previous studies of $GABA_A$ receptors demonstrating that the homologous mutation removes block by picrotoxinin (Gurley et al., 1995). This mutated subunit produced receptors that showed a significantly shifted activation curve by glycine (Fig. 1). Accordingly, the actions of drugs were tested on responses elicited by 10 μM glycine. This mutation removed block by 100 μM picrotoxinin (response in the presence of picrotoxinin was 1.8 ± 0.4 times the response in the absence, three cells). This response did not differ significantly from no effect (that is, a relative response of 1.0; $P = .1$ for difference to 1), although it is reminiscent of the observation that some mutations in the α1-subunit may render picrotoxinin a potentiator of responses to glycine (Lynch et al., 1995). Block by picrotin was removed by this mutation (Table 1). Interestingly, block by αEMTBL was also removed (Table 1).

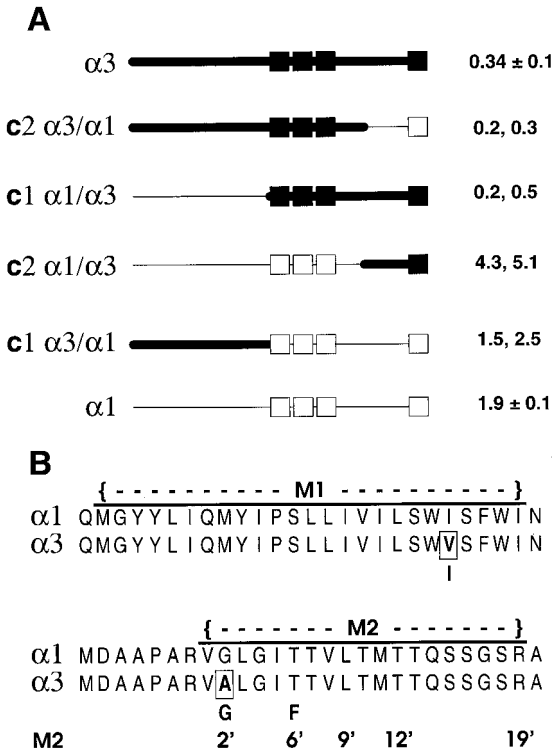

Fig. 4. Structural basis for the subunit specificity of αEMTBL action. A, graphics of the chimeric subunits constructed (see *Materials and Methods*), with the fractional response in the presence of 3 mM αEMTBL shown to the right of each line. The structure is represented with the N terminus to the left, whereas the four boxes show (from left to right) the relative positions of the M1, M2, M3, and M4 membrane-spanning regions. The joining site for c1 lay between residues 216 and 217 (in both subunits), whereas the joining site for c2 lay between residues 340/341 (α1-subunit) and 356/357 (α3-subunit). Portions derived from the α3-subunit are shown by a thick line and filled boxes, whereas those derived from the α1-subunit are shown by thin lines and empty boxes. Note that the ability of αEMTBL to block responses is associated with chimeras that contain the region of the α3-subunit, which includes the membrane-spanning regions M1, M2, and M3. Two oocytes injected with each chimera were tested for the action of 3 mM αEMTBL on responses elicited by 100 μM glycine. B, aligned sequences for the human α1 subunit (top) and the rat α3-subunit (bottom). The region shown begins at residue 218 and ends at residue 272 for both subunits and includes the M1 region (top set) and the M2 region (bottom set). The residues that differ between α1 and α3 are enclosed in boxes and shown in bold type, and the mutations made in the α3-subunit for this study are shown on the line below. The bottom row shows the M2 residue numbering used under *Discussion*. The α1- and α3-subunits are identical in sequence between the site for c1 and the region shown. They are also identical from the region shown through M3 and into the cytoplasmic region. However, there are several amino acid differences further on in the cytoplasmic region before the c2 site (not shown).

Discussion

An anticonvulsant thiobutyrolactone, αEMTBL, has opposite effects on α1 and α3 homomultimeric glycine receptors expressed in *Xenopus* oocytes. The subunit specificity can be explained by a single amino acid difference between the two subunits, located in the M2 region. It is likely that this residue is responsible for conferring the blocking action of αEMTBL and that both α1 and α3 receptors can be potentiated by a separate mechanism.

Block by αEMTBL and Picrotin. Block by αEMTBL has not been studied previously because α-substituted alkyl-butyrolactones potentiate responses of $GABA_A$ receptors. However, β-substituted compounds block $GABA_A$ receptors (Holland et al., 1990). The steady-state block produced by β-ethyl-β-methyl-γ-butyrolactone (βEMGBL) is reduced at high GABA concentrations, although the rate of block is increased (Yoon et al., 1993), suggesting a relatively complicated kinetic mechanism. Similarities in the blocking mechanisms of lactones and picrotoxinin are suggested by the finding that block by βEMTBL is removed in recombinant $GABA_A$ receptors that have point mutations which remove block by picrotoxinin [see below and Williams et al. (1997)]. Finally, it has been shown that a sulfhydryl-reactive reagent can irreversibly inactivate $GABA_A$ receptors expressed in *Xenopus* oocytes, which contain the point mutation $GABA_A$ α1 V257C [see Xu et al. (1995); this residue aligns with glycine receptor α3 A254]. Picrotoxinin (100 μM) blocked the response to GABA and also protected the receptor from inactivation, suggesting that the site was occluded by picrotoxinin. In contrast, βEMGBL does not protect the receptor from inactivation. However, when βEMGBL was added with picrotoxinin, the block produced by picrotoxinin was reduced and the receptor could also be inactivated (Xu et al., 1995). Apparently, lactones do not occlude V257 in the $GABA_A$ α1-subunit, but somehow prevent picrotoxinin from occluding the residue. Taken together the observations show that there are interactions between lactones and drugs, which act at the picrotoxinin binding site, and suggest that the interactions are mediated by an allosteric mechanism. These observations are consistent with the present finding that block of glycine α3 receptors by αEMTBL partially occludes block by picrotin.

Picrotoxinin has at least two mechanisms by which it blocks receptors. One mechanism appears to be predominant for $GABA_A$ receptors. Block develops more rapidly in the presence of GABA than in its absence, and the degree of block is greater at higher concentrations of GABA. This could re-

flect an "open channel block" in which picrotoxinin selectively interacts with an open channel (Inoue and Akaike, 1988), but picrotoxinin could stabilize a desensitized state of the $GABA_A$ receptor or induce a conformational change that alters activation by GABA (Inoue and Akaike, 1988; Newland and Cull-Candy, 1992; Yoon et al., 1993). A second mechanism of block appears to be competitive with agonist, in that block is reduced at higher concentrations of agonist. This competitive mechanism is the major mechanism of block of receptors composed of wild type ρ1 ($GABA_C$) subunits (Wang et al., 1995; Zhang et al., 1995) and of wild type glycine α1 (Lynch et al., 1995) and α3 (present results) subunits.

Structural Studies of Picrotoxinin Block. The idea that picrotoxinin interacts directly with the ion channel receives circumstantial support from observations that mutations of residues in the M2 (channel lining) helix can greatly reduce the blocking ability of picrotoxinin. However, it should be noted that many mutations in this region also affect channel conformational changes (activation or desensitization), as indicated below. For convenience, the position of a residue in the aligned M2 segments will be indicated by using an offset residue number (e.g., A254 is abbreviated by A2′; see Fig. 4).

Initial studies of glycine receptors implicated residues in the M2 region (Pribilla et al., 1992). Subsequent work with $GABA_A$ subunits demonstrated that the mutation $GABA_A$ β2 T6′F could remove block by picrotoxinin (Gurley et al., 1995) even when expressed with wild type α1- and γ2-subunits. Expression of the mutated β2-subunit with wild type α1- and γ2-subunits resulted in little change in activation by GABA. Mutations of the conserved residue L9′ also reduced the efficacy of picrotoxinin block as well as producing major changes in activation by GABA (Tierney et al., 1996; Chang and Weiss, 1998), whereas mutation of T12′ in the α1- or β1-subunits produced little effect on picrotoxinin block (Birnir et al., 1997). It is slightly perplexing that identical changes in homologous residues (T6′F) produce similar effects on picrotoxinin block in both $GABA_A$ and glycine receptors, because the block is noncompetitive in one case and apparently competitive with agonist in the other (see above). Additional study will be required to resolve this question, although one explanation is that the mechanism of block is allosteric in both cases.

Studies of a GABA-activated receptor subunit from *Drosophila* (rdl) have shown that a point mutation, rdl A2′S, confers resistance to picrotoxinin (Ffrench-Constant et al., 1993). Analysis of the function of rdl A2′S receptor has shown that several alterations in activation by GABA also occur (Zhang et al., 1994). Studies of ρ ($GABA_C$) subunits have implicated residues at positions 2′ and 6′ in determining picrotoxinin block (Wang et al., 1995; Zhang et al., 1995). The mutation which was analyzed in the present study, glycine α3 A2′G, did not remove block by picrotin, although it did remove block by αEMTBL.

In addition to the present results from glycine receptors, two mutations in the glycine α1-subunit of a residue at the outermost end of the M2 region (R19′L or R19′Q; see Fig. 4) have major effects on picrotoxinin block. Picrotoxinin potentiated responses to low concentrations of glycine from these mutated subunits, whereas at high concentrations of glycine picrotoxinin acted as a noncompetitive inhibitor (Lynch et al., 1995). These findings were interpreted to indicate that picrotoxinin is an allosteric blocker of the glycine receptor. It is interesting that for receptors containing α1 R19′L or R19′Q the EC_{50} for activation is shifted by more than 100-fold to higher glycine concentrations (Lynch et al., 1995), whereas for receptors containing α3 T6′F the EC_{50} is shifted by 10-fold to lower glycine concentrations.

Open Channel Block of Glycine Receptors. Previous studies of glycine receptors have shown that the residue in the 2′ position is critical in determining the ability of cyanotriphenyl borate (CTB) to block currents (Rundstrom et al., 1994). CTB acts as a negatively charged open channel blocker of α1 receptors, because block is greater when higher concentrations of glycine are used to activate and greater when the membrane potential is less negative. No block is seen with α2 receptors (which have alanine at the 2′ position), and block is removed from α1 receptors by the point mutation α1 G2′A (Rundstrom et al., 1994). It is interesting to note that block by CTB is noncompetitive with glycine, whereas αEMTBL is competitive. Because the sensitivity to the nature of the residue at the 2′ position is also inverted for the two drugs, it is very likely that the mechanisms of block are distinct. However, the residue at the 2′ position is clearly critical in both blocking mechanisms.

Relationship of the Mutated Residues to Channel Structure. Studies of nicotinic and $GABA_A$ receptor subunits have shown that the residues at the 2′ and 6′ positions are accessible from the channel lumen (Akabas et al., 1994; Xu and Akabas, 1996). In the nicotinic receptor the 2′ and 6′ residues are involved in ion permeation (Cohen et al., 1992; Villarroel et al., 1992), whereas the 2′ residues form the narrowest portion of the pore (Villarroel et al., 1992). The 6′ residue forms part of the binding site for some open channel blocking drugs (Charnet et al., 1990), and can be photolabeled by some noncompetitive competitors (Giraudat et al., 1987; White and Cohen, 1992). Finally, mutation of the 6′ residue in the nicotinic α7-subunit converts dihydro-β-erythroidine from an antagonist to an agonist and shifts the activation curve for acetylcholine by about 100-fold to lower concentrations (Devillers-Thiery et al., 1992). These results demonstrate that permeating ions and/or drugs can interact directly with residues in these positions, and, in addition, that the residues can have a major role in allosteric transitions of the receptor.

The data available for channels with alanine or glycine at the 2′ position show that this substitution has little effect on ion conductance (Cohen et al., 1992) or ionic selectivity (Rundstrom et al., 1994). This suggests that there is little difference in steric factors between the two residues at the 2′ position.

Conclusions

The residue at the 2′ position of the M2 region of the glycine receptor determines the subunit specificity of the action of αEMTBL. However, the blocking action involves other residues in the M2 region, and may have features in common with the action of picrotin (and picrotoxinin). The data suggest that block occurs by an allosteric mechanism. Potentiation apparently requires residues in other regions of the glycine receptor. We are currently pursuing experiments with the goals of identifying the regions critical for potentiation by butyrolactone derivatives and assessing the possi-

bility that glycine receptors are a likely target involved in their anticonvulsant actions.

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