MOLECULAR PHARMACOLOGY, 23:511-518

Computer-Assisted Modeling of the Picrotoxinin and γ-Butyrolactone Receptor Site

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Received July 12, 1982; Accepted October 12, 1982

SUMMARY

Three-dimensional models of the picrotoxinin and alkyl-substituted γ-butyrolactone (GBL) receptor sites were constructed with the aid of a molecular graphics computer system (MMS-X). These two independently derived models proved to be very compatible, which suggested that both types of compounds share a common site of action. Since picrotoxinin is known to act at γ-aminobutyric acid-regulated chloride channels, a hypothesis was made and tested that the convulsant GBL and picrotoxinin analogues physically impede the passage of chloride ions through the channel. It was also shown that it was theoretically possible for the anticonvulsant GBLs to act at this same site without blocking chloride flux. Finally, the model was applied to several convulsant and anticonvulsant compounds of different chemical classes and was found to be of somewhat general applicability.

INTRODUCTION

Picrotoxin is a sesquiterpene produced by plants of the *Menispermaceae* family. It is a potent convulsant agent whose pharmacological effects were known for centuries but were first described in detail in 1875 (1). Since then much interest has been given to this drug (for a review see ref. 2). It is now generally agreed that picrotoxin exerts its effects via blockade of the actions of the inhibitory neurotransmitter, GABA.[5] Picrotoxin blocks both the GABA-induced increase in chloride flux (3) and the resultant change in membrane potential (4). However, it does not inhibit the binding of GABA to its receptor (5). These observations led to the suggestion that picrotoxin inhibits the GABA receptor-ionophore system at a site other than the GABA recognition site. This picrotoxin site appears to be related to macromolecules that regulate chloride permeability.

Chemically, picrotoxin is actually a mixture of two substances in an equimolar ratio, picrotoxinin and picrotin. Picrotoxinin is the more active of the two, being almost 50 times more potent than picrotin (6). However, the chemistry of these compounds has a much shorter history than the pharmacology, and the correct structures were not determined until the 1950s (for a review see ref. 7).

Over 20 years ago, Enders *et al.* (8–10) reported the convulsant activity of a new group of central-stimulating substances, the alkyl-substituted GBLs. Later, Ezhov *et al.* (11) examined the bioactivity of the compounds used in the studies by Enders *et al.* in terms of the Hansch model, which correlates potency to lipophilicity. Recently, we reported a study of the structure-activity relationships of a wide variety of alkyl-substituted GBLs (12–14). Our results confirmed the convulsant activity of the β-substituted GBLs described by Enders *et al.* (8) and also demonstrated that compounds substituted with small alkyl groups in the α - and/or γ-position, but not in the β-position, had anticonvulsant activity. We proposed, therefore, that it was the β-alkyl groups which were the "effector" moiety of the convulsant GBLs. Certain structural and pharmacological similarities between the alkyl-substituted GBLs and picrotoxinin were also observed (14). For example, picrotoxinin contains a β-alkyl-substituted GBL moiety which is essential for activity (6). These findings led to the suggestion that alkyl-substituted GBLs may work at the same site as picrotoxinin.

In order to define more precisely these interesting structure-activity relationships of alkyl-substituted GBLs and to compare them with the structural requirements of picrotoxinin and related compounds, we undertook the following study utilizing an interactive real-time

This work was supported in part by National Institutes of Health Grants NS-14834, GM-07200, and GM-24483.

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[5] The abbreviations used are: GABA, γ-aminobutyric acid; GBL, γ-butyrolactone; PTZ, pentylenetetrazol; REV, receptor-essential volume; ESM, ethosuximide; TMSM, tetramethyl succinimide; CHGBL, *spiro*-cyclohexyl-GBL; CPGBL, *spiro*-cyclopentyl-GBL.

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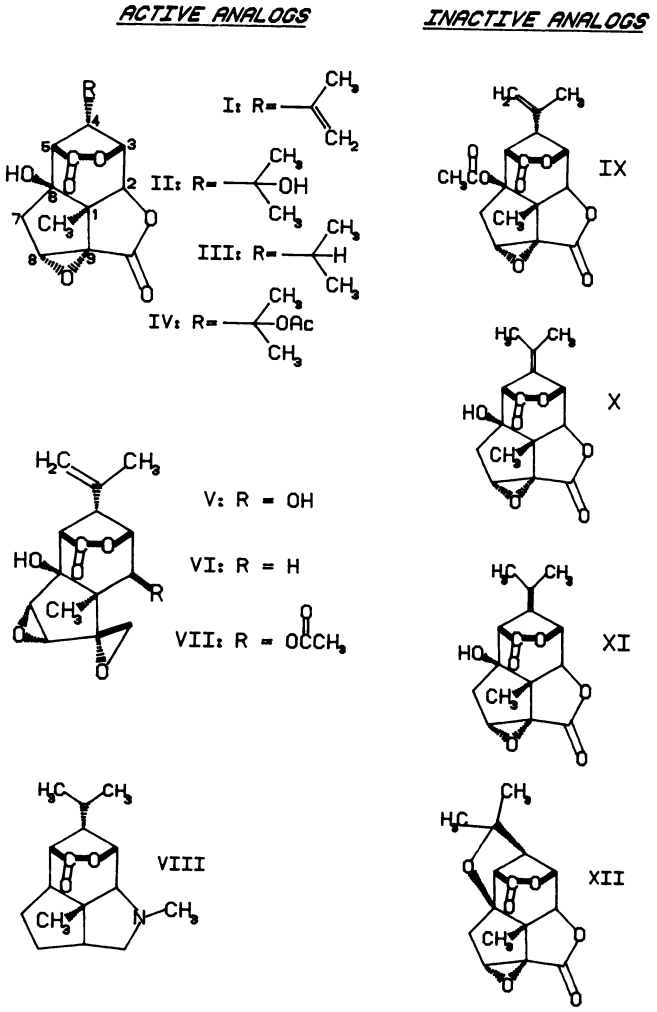
molecular graphics system (MMS-X) developed at Washington University (St. Louis, Mo.) (15).

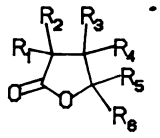
METHODS

Choice of compounds. The activities of most of the compounds in the picrotoxinin series included in this study were previously determined by Jarboe *et al.* (6). Activity was defined as the ability to produce convulsive seizures in mice. The requirements for inclusion in this study were as follows: (a) the presence of an intact lactone bridge between carbon atoms 3 and 5 of the cyclohexane ring (for numbering see Scheme 1), since Jarboe *et al.* (6) found this to be a necessary structural element; and (b) the presence of chemical stability.

On the basis of this latter requirement, α-dihydropicrotoxinin acetate was excluded because of the ease with which it is hydrolyzed to α-dihydropicrotoxinin (6). Compounds I–VII, IX, X, and XII in Scheme 1 were those compounds from the study by Jarboe *et al.* (6) which fulfilled our requirements. In addition, we included dendrobine (Compound VIII) on the basis of studies by Chen and Chen (16) and Chen and Rose (17).



SCHEME 1. *Structures of active and inactive picrotoxinin analogues* *Dashed lines* represent bonds behind the plane of the paper, and *heavy lines* represent bonds in front of the plane of the paper. Numbering according to Jarboe *et al.* (6).



COMPOUND	R_1,R_2	R_3,R_4	R_5,R_6	ACTIVITY
1	H,H	Me,Me	H,H	Convulsant
2	H,H	Et,Me	H,H	"
3	H,H	Et,Et	H,H	"
4	H,H	Pr,Pr	H,H	"
5	Me,Me	Me,Me	H,H	"
 6	H,ring	H,ring	H,H	"
 7	H,ring	H,ring	H,H	"
 8	H,ring	H,ring	H,H	"
9	Me,Me	H,H	H,H	Anticonvulsant
10	Et,Me	H,H	H,H	"
11	Et,Me	H,H	Et,Me	"
12	H,H	H,H	Et,Me	"
13	Ø,Ø	H,H	H,H	Inactive
14		H,H	H,H	"

SCHEME 2. *Structures of GBL analogues included in this study*

β-Dihydropicrotoxinin (Compound XI) was synthesized using the method of Slater and Wilson (18). The product was recrystallized from ethanol/water and had a melting point of 255°–256° [lit, m.p. 256° (18)]. The infrared spectrum showed maxima at 3450 (hydroxyl), 1805 (lactone), and 1760 cm^{-1} (lactone). The proton magnetic resonance spectrum showed a doublet of doublets at δ 0.97 (isopropyl group), and the remainder of the spectrum was also consistent with the proposed structure. The R_F on thin-layer chromatography in chloroform/methanol (95:5) was 0.36 [compared with picrotin (0.26), picrotoxinin (0.45), and α-dihydropicrotoxinin (0.51) in the same system]. Compound XI was injected i.p. as a suspension in 30% propylene glycol into female Swiss-Webster mice and was inactive in doses as high as 400 mg/kg.

In the GBL series (Scheme 2), Compounds 1, 2, 5–7, 9–11, and 14 were synthesized and tested as previously described (12–14). Compounds 12 (γ-ethyl-γ-methyl-GBL) and 13 (α,α-diphenyl-GBL) were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Compounds 3 and 4 were included in this study because of their convulsant activity in the study by Enders *et al.* (8). Compound 8 was synthesized by Diels-Adler addition of maleic anhydride to *trans*-2, *trans*-4-hexadiene (Aldrich Chemical Company) followed by sodium borohydride reduction as previously reported for Compound 6 (12). The product had a boiling point of 132°–134° (0.5 mm Hg), maximal absorption in the infrared spectrum at 1770 cm^{-1} (lactone), and absorptions in the proton magnetic resonance spectrum at δ 1.1 (doublet, CH_3), 1.3 (doublet, CH_3), 3.7–4.4 (complex multiplet, γ-lactone protons), 5.7 (doublet of quartets, vinyl protons), and 2.1–3.0 (broad complex multiplet, four remaining protons). Com-

pound 8 produced epileptiform discharges in paralyzed-ventilated guinea pigs at 3 mg/kg. This procedure has been described previously (12).

Steric analysis. A preliminary conformation of picrotoxinin (Compound I) was generated by modification of α_1-bromopicrotoxinin, whose Cartesian coordinates were obtained from X-ray crystallographic determinations (19). Following this modification, the least-energy conformation of picrotoxinin was determined by the strain-energy minimization procedure, MM-2, of Allinger and Yuh (20). A systematic conformational search around all four rotatable bonds (i.e., the isopropenyl group at C-4, the isopropenyl methyl, the hydroxyl at C-6, and the methyl at C-1) yielded only two distinct conformations under 2.0 kcal in energy above the minimum. These differed only by 186° rotation of the isopropenyl group. The minimum had the double bond toward the α carbon atom of the lactone; the other, 1.5 kcal higher in energy, had the double bond toward the γ carbon atom of the lactone.

This picrotoxinin molecule was then modified as necessary to produce Compounds II–XII, and the resulting structures were again submitted to the minimization program. In addition, conformational searches were performed as required. Only one unique low-energy (<2.0 kcal from the minimum) conformation was found for each molecule except Compound VII, in which 120° rotation of the acetate group was allowable in this energy range. In this case, all three conformations were used in further manipulations.

The compounds in Scheme 2 and Fig. 4 were built from standard fragments and minimized as described above.

It is important in this kind of study to have a consistent alignment of the molecules in 3-dimensional space. This was accomplished by the use of a least-squares fitting program using picrotoxinin as the reference for all molecules. In the picrotoxinin series, the active analogues, Compounds II–VIII, were easily superimposable on picrotoxinin using the atoms of the lactone, cyclohexane, and cyclopentane rings. The inactive analogues, Compounds IX and XI, were also aligned this way. Since Compounds X and XII have slightly distorted isopropenyl groups, they were fitted as above and also using only the lactone oxygen atoms and isopropenyl carbon atoms. Both orientations of Compounds X and XII were used in computing the model.

In the GBL series, and for the comparisons made in Fig. 4, each compound was fitted so that its lactone oxygen atoms (or imide nitrogen and oxygen atoms) overlapped as closely as possible those of picrotoxinin. PTZ was oriented so that its tetrazol ring overlapped the lactone ring of picrotoxinin, disregarding the carbonyl oxygen atom. All of these molecules were then tilted either forward or backward along the axis defined by the lactone oxygen atoms of picrotoxinin so that the best overlap of the remaining alkyl groups to the rest of the picrotoxinin molecule was obtained. Once oriented, all molecules retained the same relative coordinates throughout the study.

For each aligned molecule, an electron density volume map was generated using Gaussian functions which were surface-contoured at the level representative of van der Waals radii (21). The union of the active analogues (Compounds I–VIII in the picrotoxinin series or Compounds 1–12 in the GBL series) allowed construction of the receptor-excluded volume map, i.e., that volume which is available at the binding site as indicated by the volume requirements of the active molecules (22).

The volume occupied by the receptor in the vicinity of the binding site was estimated in the following way. In each of the picrotoxinin and GBL series, the union of the volumes of all inactive compounds was computed. Next, the part of this inactive volume which was common to the union of the active analogues was subtracted. This difference represents that portion of the inactive molecules which is unique. It is assumed that an inactive molecule is inactive because it cannot fit into the receptor site. Accordingly, some part of its unique volume is assumed to be essential for the receptor itself. Once determined, this REV can be used to test the "goodness of fit" of other molecules into this same binding site. This is done by determining the overlap, or intersection, of the volume of a given molecule with that of the computed REV.

RESULTS

The picrotoxin series. Figure 1A illustrates the determination of the REV (labeled *Difference*) for the picrotoxinin series. The active analogues used in this study

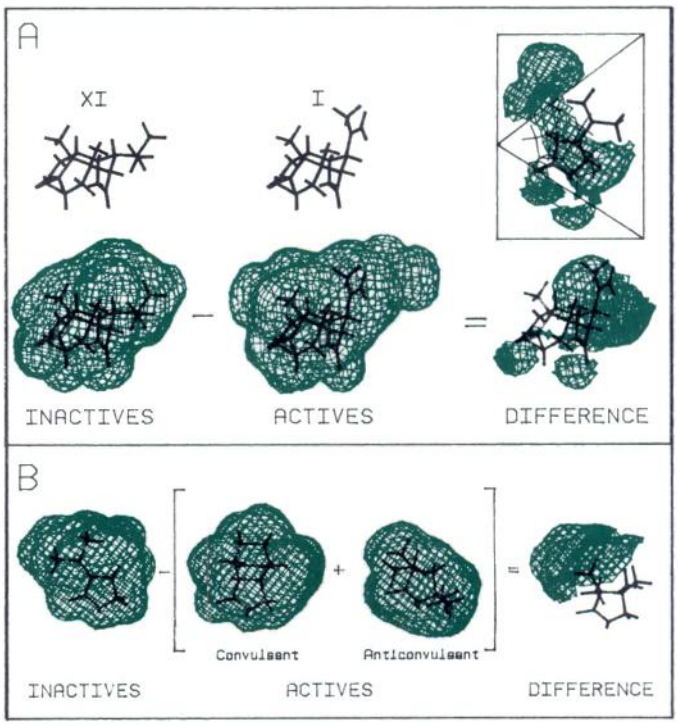


FIG. 1. *Computation of picrotoxinin REV and GBL REV*

A. Computation of the picrotoxinin REV, which is labeled *Difference*. The *upper right* shows a different view of this REV, divided to show the contributions from the individual inactive compounds (see text). The structures of β-dihydropicrotoxinin (*XI*) and picrotoxinin (*I*) are shown above and inside the volumes of the inactives and actives, respectively. Volumes shown are contoured at the van der Waals radii.

B. Computation of the GBL REV.

were picrotoxinin (*I*), picrotin (*II*), α-dihydropicrotoxinin (*III*), picrotin monoacetate (*IV*), tutin (*V*), coriamyrtin (*VI*), tutin monoacetate (*VII*), and dendrobine (*VIII*). The inactive analogues used were picrotoxinin acetate (*IX*), neopicrotoxinin (*X*), β-dihydropicrotoxinin (*XI*), and anhydropicrotin (*XII*). The minimized, computer-generated structures of picrotoxinin (*I*) and β-dihydropicrotoxinin (*XI*) are shown both above and inside the union of the volumes of the active and inactive analogues, respectively. Subtraction of the common element of the actives and inactives from the union of the inactives gives a difference which represents that volume unique to the inactive compounds. If it is assumed that it is this volume which is responsible for each inactive compound's not fitting into the receptor, then the receptor must have volume occupying at least some of the volume unique to each inactive molecule. When rotated as shown in the upper representation of this REV (Fig. 1A), the contributions of each inactive molecule can be seen separately. The *upper left* portion of the REV is from the acetate group of Compound IX. The two distinct *lowermost* portions are from the distorted backbones of Compounds X and XII. The remaining *central* portion is due largely to the isopropyl group of Compound XI (which is epimeric with that of Compound III), but is also overlapped by the similarly oriented isopropylidene group of Compound X. The section connecting the acetate (Compound IX)-derived volume in the *upper left* and the isopropyl (Compound XI)-derived volume in the *middle right* is a thin ribbon which is of uncertain importance.

The volume which was removed from the union of the inactives was determined from the union of all of the actives, but similar results could have been obtained by using only Compounds I and II, picrotoxinin and picrotin. This is not surprising, since Compounds III, V, VI, and VIII are structurally very similar to Compounds I and II and the acetate groups of Compounds IV and VII have little common volume with the inactive compounds not already accounted for by Compounds I and II.

Thus, for a compound to be active at the binding site of the picrotoxinin series of compounds, it should have no major overlap with the REV shown in Fig. 1A.

The GBL series. Figure 1B illustrates the determination of the REV for the GBL series. One difference between this series and the picrotoxinin series is that there are two types of active compounds—convulsant and anticonvulsant—as well as the inactive compounds. It was assumed in this study that both the convulsants and anticonvulsants work at the same site, and there is some evidence for this (8). The active convulsant compounds used in this study were β,β-dimethyl-GBL (1), β-ethyl-β-methyl-GBL (2), β,β-diethyl-GBL (3), β,β-dipropyl-GBL (4), α,α,β,β-tetramethyl-GBL (5), bicyclo-[2.2.1]-hept-5-ene-*cis*-2-hydroxymethyl-3-carboxylic acid lactone (6), bicyclo-[2.2.2]-oct-5-ene-*cis*-2-hydroxymethyl-3-carboxylic acid lactone (7), and 3,6-dimethyltetrahydrophthalide (8). The active anticonvulsant compounds used were α,α-dimethyl-GBL (9), α-ethyl-α-methyl-GBL (10), α,γ-diethyl-α,γ-dimethyl-GBL (11), and γ-ethyl-γ-methyl-GBL (12). The inactive compounds were α,α-diphenyl-GBL (13) and α-isopropylidene-GBL (14).

The unions of active and inactive compounds and the REV were determined in the same way as those for the picrotoxinin series and are shown in Fig. 1B, with Compound 14 inside the inactive volume, Compound 7 inside the active convulsants, Compound 11 inside the active anticonvulsants, and Compound 5 in relation to the REV. Compound 11 comprises essentially all of the anticonvulsant volume, and the convulsant volume may be largely accounted for by Compounds 4, 7, and 8 alone.

Comparison of picrotoxinin and GBL models. If the hypothesis is correct that the alkyl-substituted GBLs are working at the same site as the picrotoxinin analogues, then the two independently derived models discussed above should be complementary. To test this, we determined the overlap of the active GBLs with the picrotoxinin REV and the overlap of the active picrotoxinins with the GBL REV. The results are shown in Fig. 2. Ideally, there would be zero overlap. However, in both cases there was a slight overlap. The active GBLs just contacted the *darkened* portion of the picrotoxinin REV shown in Fig. 2 (*top right*). What cannot be appreciated in this 2-dimensional representation is that, although the surface area of this contact is large, it is very narrow and represents only a small part of this middle section of the volume. The active picrotoxinin analogues also had a small contact with the portion of the GBL REV due to one of the phenyl rings of Compound 13 (Fig. 2, *middle right*).

A combined REV was then computed by subtracting both the active GBL and picrotoxinin analogues from the combined inactive GBL and picrotoxinin analogues. The

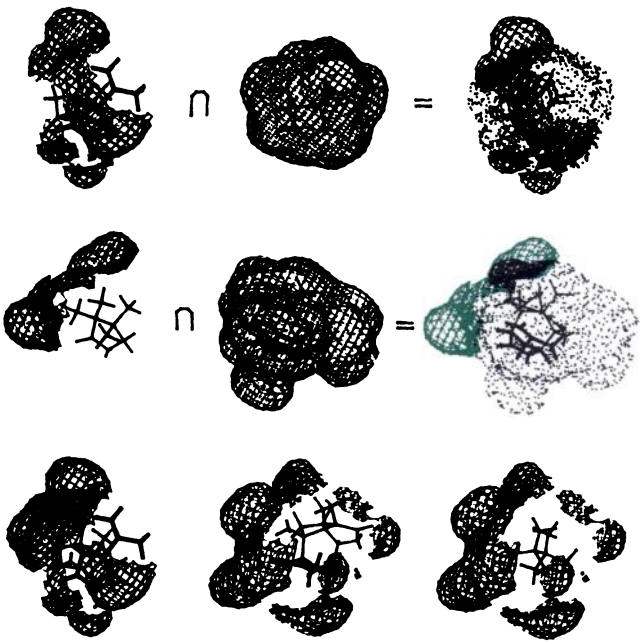


FIG. 2. *Comparison of the picrotoxinin and GBL models*
Top row. The intersection of the picrotoxinin REV (*left*) with the active GBLs (*center*) is shown as a *darkened* area of the REV (*right*). The *dotted* area (*right*) represents the active GBL volume.
Middle row. Similar intersection (*darkened* area at *right*) of the GBL REV (*left*) with the active picrotoxinin analogues (*middle*).
Bottom row. *Left*, combined GBL-picrotoxinin REV. Note similarity to original picrotoxinin REV (top row, *left*). *Middle*, Another view of this combined REV with a picrotoxinin molecule occupying the site. *Right*, Same view with a GBL (Compound 5) occupying the site.

resulting REV is shown in the *lower left* of Fig. 2 and can be compared with the original picrotoxinin REV in the *upper left* of Fig. 2. Two things can be seen. First, almost all of the original picrotoxinin REV remains and, second, the lost portion of the original picrotoxinin REV is largely replaced by the GBL REV, although it is pushed outward a small distance.

A different view of this combined REV is also shown in the *lower middle* portion of Fig. 2, with a picrotoxinin molecule oriented in the same way, and at the *lower right* with a convulsant GBL, Compound 5, also oriented in the same way.

Modeling the chloride channel. As was stated under Introduction, it is believed that the picrotoxinin site is related to the macromolecules that regulate GABA-dependent chloride permeability. The exact mechanism of this blockade is unknown, but we hypothesize that it could be due to a direct blockade of chloride flux by the picrotoxinin molecule itself. To test this idea, we constructed and oriented a chloride channel and ion model as described below.

The pertinent physical parameters of such an ionophore are the diameter of the channel itself and the diameter of the hydrated chloride ion which must pass through the channel. The data of Araki *et al.* (23) and Takeuchi *et al.* (24) are quite consistent and suggest that the largest anion which can pass freely through the chloride channel is the chlorate ion (ClO_3^-), which has a hydrated radius of 2.85 Å (23). The smallest anion which is impeded by the channel is the bromate ion (BrO_3^-), with a hydrated radius of 3.25 Å (23). Since these values are considered the upper and lower limits of the effective pore radius of the chloride channel, we arbitrarily chose the average of 3.05 Å as the radius of the chloride channel in our study (larger cylinder in Fig. 3). The radius of a hydrated chloride ion is 2.36 Å (23) (smaller cylinder).

The reasons for our choice of orientations of the chloride ion and channel with respect to the REV and molecules require some explanation. As was noted in a previous report (14) and can be seen in Scheme 2, the most notable difference between the convulsant and anticonvulsant GBLs is the lack of alkyl groups at R_3 and R_4 (the β-position) of the anticonvulsants and the presence of alkyl groups here in the convulsants. This fact led us to suggest that it is the substituents at this position which are responsible for the activity of the compound. Picrotoxinin also has a GBL ring with an isopropenyl group at the β-position. We hypothesize that it is this group which actually blocks Cl^- flux and, if it were replaced by a hydrogen atom, chloride flux would not be impeded. Therefore, the chloride channel model was centered along the vector of the bond from the β-carbon atom of the picrotoxinin GBL ring to the middle carbon atom of the isopropenyl group, as is shown in Fig. 3 (*top left*). The distance of the channel away from the picrotoxinin molecule was chosen to be that distance at which the van der Waals radii of a hydrated chloride ion would just not touch the van der Waals radii of a picrotoxinin analogue, with the isopropenyl group replaced by a hydrogen atom as shown in the *top middle* of Fig. 3. To make this situation correspond to a "just open" channel, the chloride ion was displaced to the far side. Inhibition of chloride flux would appear in this model as an overlap of the van der Waals radii of a molecule with that of the hydrated chloride ion (the inner circle).

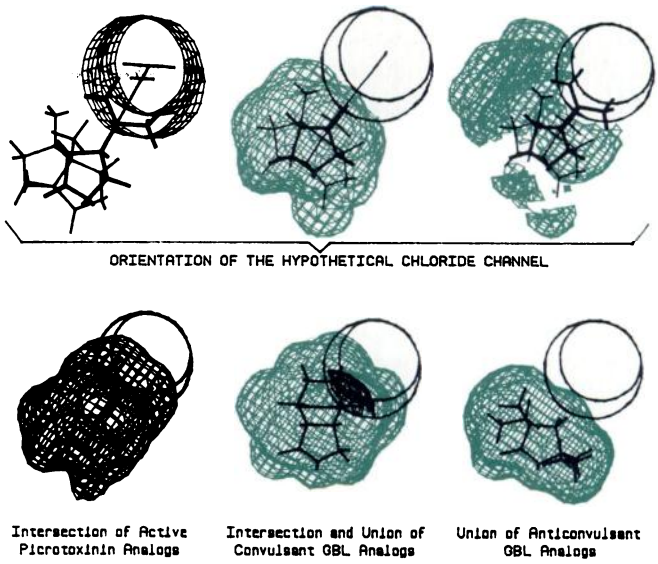


FIG. 3. *Orientation and testing of the chloride channel model* *Top, left,* The channel (larger cylinder) was oriented perpendicular to the vector running from the β-lactone carbon atom of picrotoxinin to the center carbon atom of the isopropenyl group. *Top, center,* The distance along this vector was chosen so that the chloride ion (inner cylinder) could just pass a picrotoxinin analogue whose isopropenyl group had been replaced by a hydrogen atom. *Top, right,* The chloride channel model in relation to the combined REV. *Bottom, left,* The channel model shown with the intersection of the volumes of all active picrotoxinin analogues. Note overlap of this volume with that needed by the chloride ion. *Bottom, middle,* Channel shown with union of convulsant GBLs. Note that the overlap of the chloride ion is similar to that of the picrotoxinin analogues. The *darkened* area represents a segment of the smallest GBL, Compound 1. *Bottom, right,* Channel with union of anticonvulsant GBLs. Note lack of overlap with the chloride ion.

While keeping the above orientation, this channel-ion model was compared with several previously determined volume elements. First, it can be seen (Fig. 3, *top right*) that the channel does not intersect the combined picrotoxinin-GBL REV. This view is from what would correspond to being inside a cell, looking out of the channel, and the small segment of the REV which appears to intersect the left side of the channel is behind it (i.e., jutting up from the exterior of the cell surface) and would not hinder the entrance of a chloride ion into this hypothetical channel. This lack of intersection is important for consistency of the two models, since the REV specifies volume occupied by the receptor and the channel specifies a normally empty volume.

Next it can be seen (Fig. 3, *bottom left*) that all of the active picrotoxinin analogues would block the entrance of the chloride ion in this model. The volume shown here represents the intersection of all of the active picrotoxinin analogues, so the extent to which even the smallest analogue overlaps the chloride ion is depicted. This is important, since all these active convulsants are assumed to inhibit chloride flux.

In the GBL series, there are both convulsants and anticonvulsants. We hypothesized that the convulsants

work to inhibit chloride flux in the same manner as picrotoxinin on the basis of structure-activity relationships (14). These convulsants overlap the chloride ion in our model in much the same way as did the picrotoxinin analogues (Fig. 3, *bottom center*). The small *darker* volume represents the overlap of the smallest GBL, Compound 1, with the chloride ion. The larger volume represents the union of all of the convulsant GBLs and is more representative of Compounds 2–8.

We also proposed (14) that the anticonvulsant GBLs could act at the same site as the convulsant GBLs and picrotoxinin but would have no chloride-blocking ability of their own and, as a result, would show anticonvulsant activity. The lack of alkyl groups at the β-position (R_3 and R_4 in Scheme 2) was taken to explain the lack of convulsant activity. The present model is consistent with this idea (Fig. 3, *lower right*). Shown is the union of all of the anticonvulsant GBLs, Compounds 9–12. No portion of the anticonvulsant volume intersects the chloride ion. Thus it could enter the channel freely in this model.

Application of the model to other compounds. The above model can be applied to certain other classes of convulsant and anticonvulsant compounds, such as the succinimides, glutarimides, and tetrazols (Fig. 4). Two representative succinimides, the anticonvulsant ESM and the convulsant TMSM, fit the model well. The *darkened* areas of the volume map represent the areas of overlap with the combined GBL-picrotoxinin REV (*dotted* areas). ESM has only negligible contact near the imide nitrogen atom. TMSM has this contact as well as a minor overlap of one of its methyl groups. In addition, ESM does not intersect the chloride ion volume whereas TMSM does. This is consistent with the anticonvulsant activity of ESM and the convulsant activity of TMSM (13, 14).

The classical experimental model of petit mal (absence) epilepsy is the PTZ-induced seizure (25). PTZ-induced seizures show many similarities to those induced by picrotoxinin (2). In addition, this drug has been shown to inhibit chloride conductance selectively in *Aplysia* neurons (26). It was of interest, therefore, to see whether PTZ could fit this model. When the tetrazol ring of PTZ is positioned like the GBL rings of picrotoxinin, only a thin patch of contact with the combined GBL-picrotoxinin REV is seen in the area of the third methylene group of the ring (Fig. 4). Even this contact can be largely eliminated by greater folding of the relatively flexible 7-membered ring. It should also be noted that PTZ has a significant overlap with the chloride ion, consistent with its inhibitory activity on chloride conductance (26).

The anticonvulsant glutarimide, glutethimide, has only marginal fit, with two significant areas of contact, but does not overlap the chloride ion. Perhaps glutarimides with only smaller alkyl groups (such as ethyl or methyl) would fit this model better. In any event, glutethimide does not intersect the total unique volume of any inactive compound. The convulsant glutarimide, bemegride, has only ethyl and methyl substituents, on the β-position, and fits the model extremely well. Again, this convulsant shows considerable overlap with the volume needed by the chloride ion in our model.

This model may also explain some of the results of

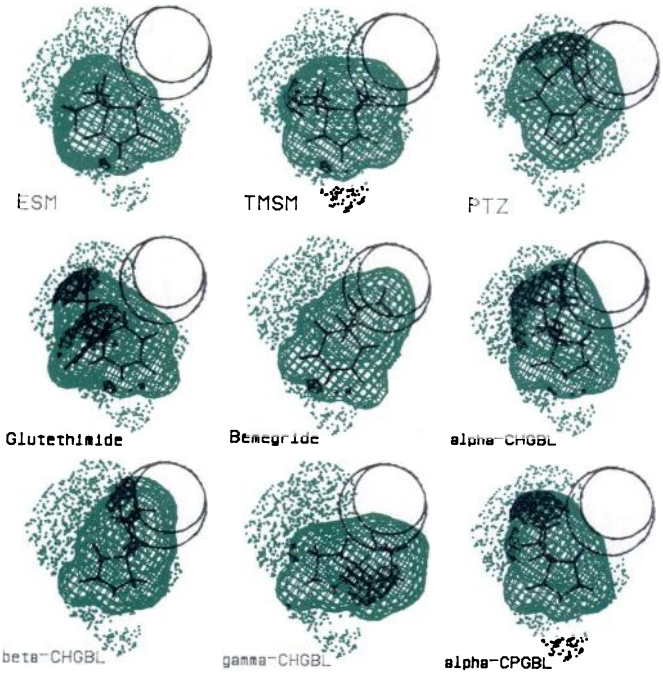


FIG. 4. *Application of the combined GBL-picrotoxinin REV (dotted area) to other classes of compounds*

The volume of each compound is shown in *solid lines* with the intersection with the REV, shown as a *darkened* area. None of these contacts represents the total volume of any inactive molecule. Note that only the convulsant compounds intersect the chloride ion volume.

Enders *et al.* (8), who found α-, β-, and γ-*spiro*-cyclohexyl GBLs to be convulsant whereas an α-*spiro*-cyclopentyl GBL was not. Being alkyl-substituted GBLs, it is not surprising that none of these compounds has a very large volume which overlaps that of the GBL-picrotoxinin REV (Fig. 4). In addition, all of the convulsant *spiro*-cyclohexyl GBLs overlap the chloride ion volume. However, the α-CPGBL does not overlap this area (Fig. 4). Enders *et al.* (8) found that this compound did not cause seizures but did not test it for anticonvulsant activity. The role which ring conformation plays in these GBLs is discussed below.

DISCUSSION

The study by Jarboe *et al.* (6) pointed out the structural specificity required at the site responsible for the convulsant activity of picrotoxinin analogues. We have recently observed (12–14) a similar specificity for alkyl-substituted GBLs. The present study extends these observations by defining and directly comparing these steric criteria in three dimensions with the aid of a molecular graphics computer system (15).

Several things should be pointed out about the choice and manipulation of compounds included in this study. With an assay for activity as nonspecific as convulsant activity in mice, one must be careful to include only structurally similar compounds that are likely to be working at exactly the same receptor. For example, bicuculline is structurally very different from picrotoxinin and also causes convulsive seizures in mice. However, this drug is known to act at a different site and, therefore, was not

included. Similarly, inactive compounds must be structurally very similar in order for their unique volumes to have meaning as an REV. Compounds such as atropine, which also does not produce seizures, would be inappropriate to this study because there is no obvious way to orient such a compound: any unique volume obtained would define only space which lies outside of that allocated to the active compounds and would carry little or no weight in defining space occupied by the receptor. Likewise, inactive picrotoxinin derivatives, without lactone rings, were not included since it is believed that lactone rings are a necessary moiety for action at this site (6). In contrast, an inactive compound such as β-dihydropicrotoxinin (Compound XI), which is structurally similar to picrotoxinin in every way except for the orientation of the isopropyl group, is most likely inactive because some of the unique volume required by this isopropyl group is occupied by the receptor itself.

The reason for this "partial occupation" of the unique volume lies in the limited number of active analogues. If every possible point in space available at the receptor site were accounted for in the group of active analogues studied, then the unique volume of an inactive compound would be assumed to be entirely occupied by the receptor. Since this is not the case, we can only assume that part of the unique volume of an inactive compound is occupied by the receptor. This raises the following question: How much of the REV model does a "test" compound have to overlap before it should be considered a bad fit? The most rigid interpretation would be that only compounds which overlap the entire unique volume of any one of the inactive compounds would be a certain bad fit. Beyond this, it would seem reasonable to assume that the portion of the REV furthest away from the volume of the active compounds is that which would most likely be occupied by the receptor. Thus, thin areas of contact which do not extend far into the REV (such as that seen between the active GBLs and the picrotoxinin REV in Fig. 2) are much less bothersome, even if they are extensive, than one which would extend completely through the REV. In this study, all of the contacts shown in Figs. 2 and 4 are of the former type, and none of these overlaps the majority of the unique volume of any single inactive compound. Not shown here are contacts of this REV with molecules such as bicuculline, which are known not to act at the picrotoxinin site. These molecules have vast overlaps which extend through the REV in several places.

We believe that the fit of the active GBLs and picrotoxinin analogues into the REVs of each other is sufficiently good to suggest that both could work at one common site. In addition, several other convulsant and anticonvulsant classes of compounds, most notably the succinimides, also fit this combined GBL-picrotoxinin model well. If this is so, then one must account for the finding that some of these compounds are convulsant and others anticonvulsant.

To explain this observation, we have attempted to use our model to help understand the primary mechanism of picrotoxinin action, blockade of chloride flux. There are two distinct mechanisms by which a drug such as picrotoxinin could block ionic flux. The first involves the classical schema of the picrotoxinin ligand binding to its receptor at a site distant from the ion channel and inducing a conformational change which would then indirectly block chloride flux. Unfortunately, although such explanations have become standard, they are usually too vague to give any insight into the physicochemical basis of drug action. Another explanation would be that all or part of the picrotoxinin molecule itself physically blocks the passage of chloride ions. This "drain plug" type of explanation has precedent in the widely accepted mechanism of tetrodotoxin blockade of the voltage-dependent sodium channel (27), and we believe that this could also explain the action of picrotoxinin. There is also some evidence that picrotoxinin competes directly with the chloride ion (28).

Direct testing of the mechanism of chloride channel blockade will most likely await purification and X-ray crystallography of the picrotoxinin-ionophore complex. At present, we could test this hypothesis only by comparing the structure-activity data obtained from picrotoxinin and related compounds with the physical parameters of the chloride channel. When this was done, it was found that the dimensions of the chloride channel and chloride ion are such that it was possible to orient them so that the convulsant β-substituted GBLs always occupied some of the volume needed for passage of the chloride ion whereas the anticonvulsant GBLs never did. This also holds true for compounds of several other chemical classes. This model gives a physical basis for the difference in pharmacological activities of the convulsant and anticonvulsant compounds. Although both types of compounds would bind at the same site and with the same orientation, the β-substituted convulsants would block chloride flux whereas the non-β-substituted anticonvulsants would not. This latter effect could either stabilize chloride flux at normal levels or augment it if there were other agents present (either exogenous or endogenous) that would block the chloride channel.

It must be stressed that the chloride channel described here refers only to the subtype associated with GABA receptors. Other chloride channels exist which are regulated by other neurotransmitters such as glycine. These various channels appear to be very similar in size and shape. Furthermore, drugs such as tutin (29), bicuculline (30), and strychnine (30) have been shown to affect both the GABA and glycine systems. There are major differences between these two inhibitory systems, however, and the results of the present study specifically concern only the GABA-regulated chloride channel.

The chloride channel model may also explain the apparent incongruities between our studies (12–14) and those of Enders *et al.* (8). Whereas we found that GBLs substituted only at the α- or γ-positions were anticonvulsants, Enders *et al.* found α-, β-, and γ-CHGBLs to be convulsant. Enders *et al.* also found that an α-CPGBL did not cause seizures. These results seem to relate to the relative sizes of the alkyl substituents. The cyclohexyl groups are large enough to reach the area needed by the chloride ion, even from the α- or γ-position, whereas the smaller ethyl and methyl groups used in our studies never do. The cyclohexane rings must be in the twist boat conformation in the α- and γ-CHGBLs to overlap the chloride ion, while the β-compound will do this in

the more stable chair form as well. The relative abundance of these "chloride overlapping" conformations may explain the potency differences observed by Enders *et al.* They found the β-compound to have an LD_{50} of 3 mg/kg in rats; the α-compound, 30 mg/kg; and the γ-compound, 90 mg/kg (10). The smaller cyclopentyl group cannot overlap the chloride ion from the α-position even when the ring is twisted (Fig. 4).

The overlap of the volume due to the β-alkyl substituents on GBLs with the volume of the chloride ion in this model again points out the importance of this group. In fact, if the anticonvulsant volume is substracted from the convulsant volume, the only unique portion of the convulsants is that volume occupied by the β-alkyl groups. Also, recall that picrotoxinin also has a GBL ring with a β-alkyl group, the isopropenyl. Indeed, all of the convulsant picrotoxinin analogues have some variation of this β-substituted GBL ring.

Although we call the area which these β-alkyl groups overlap the "chloride ion," it would be a very important area of space even if it were something else. Thus, regardless of whether the chloride channel of the cell is oriented anywhere near the picrotoxinin site, this volume element still would retain its predictive value for the convulsant or anticonvulsant activity of proposed compounds.

In this predictive value lies the true importance of models such as the one developed here. The definition of the precise volume requirements of the GBL-picrotoxinin site would allow the exploitation of these requirements in future drug design. Indeed, this model suggests which areas are inaccessible to picrotoxinin analogues and also which areas determine whether a compound will be convulsant or anticonvulsant. This knowledge should be useful in the design of anticonvulsant picrotoxinin analogues which would have a potency as great as that of the parent compound.

ACKNOWLEDGMENTS

The authors would like to thank Dr. John P. McAlister for his excellent graphic programs and his helpful guidance in their use. We would also like to thank Dr. Garland Marshall for his advice and discussion.

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