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A molecular model for the synergic interaction between γ-aminobutyric acid and general anaesthetics

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Abstract

Within the context of the discussion about rational polytherapy, we determined the effects of four anaesthetics on the binding of [^{3}H]*t*-butylbicycloorthobenzoate ([^{3}H]TBOB) to the $GABA_A$ receptor complex in the presence of several concentrations of GABA (γ-aminobutyric acid), in order to build a molecular model that can describe and quantify the interactions between the compounds. The empirical isobole method revealed that GABA and the anaesthetics acted synergically in displacing [^{3}H]TBOB. This synergy could be described by a simple molecular model in which both GABA and the anaesthetics displaced [^{3}H]TBOB allosterically and in which GABA allosterically enhanced the binding of the anaesthetics. To get information about the interaction between GABA and anaesthetics, we used [^{3}H]TBOB as a tracer ligand. The model indicated that GABA enhanced the affinity of thiopental 3.0-fold, propofol 5.0-fold, the neuroactive steroids Org 20599 3.5-fold and Org 20549 13-fold. Insight into the molecular mechanism and strength of these interactions can help clinicians to choose therapeutically optimal drug and dose combinations: a step towards rational polytherapy.

Keywords: $GABA_A$ receptor complex; Anaesthetic; TBOB (*t*-butylbicycloorthobenzoate); Polytherapy; Synergy; Isobole method; Molecular model

1. Introduction

When the dose–effect relationships of GABA-ergic (γ-aminobutyric acid-ergic) drugs are studied, usually only the concentration of a single drug is varied. There are however clinically important reasons in favour of investigating *combinations* of these drugs as well. In the first place, in the treatment of malignant diseases and infections, combinations of agents are used because of the therapeutic advantages they provide over single agents (Berenbaum, 1989). Also in anaesthesiology the use of polytherapy is everyday practice. However, this is not the case in epileptology, where the use of polytherapy is discouraged (Reynolds and Shorvon, 1981; Lammers et al., 1995; Deckers et al., 1997). This contrast is remarkable because both in anaesthesiology and in epileptology drugs with the same molecular mechanism are used, namely GABA-ergic compounds. (Rogawski and Porter, 1990; Tanelian et al., 1993). In both areas, studies have been conducted to evaluate the pros and cons of polytherapy (e.g Bovill, 1997; Deckers et al., 1997). In many studies, however, the total amount of drug has not been taken into consideration (Deckers et al., 1997). Erroneous conclusions may be drawn if this drug load as a factor for differences in efficacy or tolerability is neglected (Deckers et al., 1997; Roks et al., 1999).

In the second place, the response to a drug is subject-dependent (Dirksen et al., 1997b). For example, even in monotherapy, the effectiveness of a drug depends on the functionality of endogenous substances that affect the excitability

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of the central nervous system, such as endogenous GABA (Dirksen et al., 1997a) and neurosteroids (Macdonald and Olsen, 1994; Joëls, 1997). Per individual, the endogenous concentrations of these substances vary (Cools et al., 1990; Dirksen et al., 1997b). Moreover, drugs prescribed for one indication, e.g., to induce anaesthesia, may interact with medicines already prescribed for other indications, e.g., for epilepsy.

The aim of our research is to investigate the effects of combinations of GABA-ergic drugs in vitro, by means of receptor binding studies (van Rijn et al., 1992). The $GABA_A$ receptor complex comprises the Cl^- channel and binding sites for several compounds, such as benzodiazepines, barbiturates, neuroactive steroids (Sivilotti and Nistri, 1991; Gee et al., 1995), and a variety of other drugs such as loreclezole (van Rijn and Willems-van Bree, 1993) and propofol (Concas et al., 1991). Furthermore, a convulsant site is present on the complex (Sivilotti and Nistri, 1991). All these binding sites are allosterically coupled, resulting in a network of interactions that ultimately regulate the permeability of the Cl^- channel (Sieghart, 1992; Sivilotti and Nistri, 1991). The functional state of the channel can be assayed by measuring the amount of ligand that binds to the convulsant site (Havoundjian et al., 1986; Wha Bin Im and Blakeman, 1990). We can therefore measure the effects of allosteric interactions that modulate GABA-ergic neurotransmission in receptor binding studies with ligands that bind to the convulsant site. [^{3}H]*t*-butylbicycloorthobenzoate ([^{3}H]TBOB) can be used as a tracer ligand (Jakubik et al., 1997) for the convulsant site (van Rijn et al., 1990; Lawrence et al., 1985).

In the present study, our question was how GABA modulates the effect of GABA-ergic anaesthetics on the binding of [^{3}H]TBOB. The allosteric interaction between the binding of GABA and an anaesthetic was evaluated from changes in the binding of [^{3}H]TBOB occurring in the presence of fixed concentrations of GABA and of increasing concentrations of anaesthetic. Four different anaesthetics were examined: two clinically used anaesthetics, thiopental and propofol, and two experimental general anaesthetics of the neurosteroid type, Org 20599 [(2β,3α,5α)-21-chloro-3-hydroxy-2-(4-morpholinyl)pregnan-20-one methanesulphonate] (Hill Venning et al., 1996) and Org 20549 [(2β,3α,5α)-21-hydroxy-3-hydroxy-2-(4-morpholinyl)pregnan-20-one methane-sulphonate].

First, we describe the data in terms of additivity using the empirical isobolic method (Tallarida, 1992), which allows only qualitative conclusions. Next, we describe the observed interactions with an allosteric three-ligand molecular model, which we present in this paper, and which allows us to quantify the interactions as well.

2. Materials and methods

2.1. Preparation of the tissue

Forebrains of Wistar rats (Cpb Wu; body weight 350 ± 50 g (mean ± S.D.)) were used. The brains were homogenised in 9 vol. 0.32 M sucrose at 0°C with a Teflon-glass homogeniser. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was decanted and centrifuged at $48{,}000 \times g$ for 30 min at 4°C. The pellets were washed two times by suspension in 50 mM sodium–potassium-phosphate buffer, pH 7.4, containing 500 mM NaCl (assay buffer) and centrifugation at $48{,}000 \times g$ for 10 min at 4°C. The pellets were frozen, thawed and washed three times in order to remove endogenous GABA. The pellets were stored at −20°C until assay. Before assay, the pellets were washed once.

2.2. Assays

The pellets were homogenised in assay buffer. The tissue concentration in the incubation medium was 12.5 mg tissue wet weight/ml. Into glass tubes we added consecutively 25 μl of [^{3}H]TBOB and either drugs or buffer in volumes of 25 up to 100 μl. The final concentration of [^{3}H]TBOB was 8 nM. The incubation was started by adding 200 μl of tissue homogenate. Incubations were performed at 25°C, lasted 90 min and were terminated by addition of 3 ml ice-cold buffer to the tubes and rapid filtration of the mixture. The filters were washed two times with 3 ml cold assay buffer. Radioactivity retained on the filters was counted by liquid scintillation spectrometry. Specific [^{3}H]TBOB binding was defined as total binding minus the remaining binding in the presence of 100 μM picrotoxin. Specific binding was 75–80% of total binding at 8 nM [^{3}H]TBOB.

The binding of [^{3}H]TBOB in the presence of 20 μM bicuculline-methochloride was determined to assess the presence of endogenous GABA (Squires and Saederup, 1987; van Rijn et al., 1992).

[^{3}H]TBOB displacement curves were made for GABA, thiopental, propofol, Org 20599 and Org 20549. The [^{3}H]TBOB displacement curves for the anaesthetics were determined in the absence of exogenous GABA and in the presence of 0.4, 1, 4 and 8 μM added GABA.

2.3. Chemicals

GABA, bicuculline-methochloride and picrotoxin were obtained from Sigma. Propofol was from Aldrich. Thiopental-sodium was from Rhône-Poulenc Rorer. The neurosteroids Org 20599 and Org 20549 were a gift from Organon Technica (Newhouse, Scotland). [^{3}H]TBOB was obtained from Amersham. The specific activity was 30 Ci/mmol.

Propofol was first dissolved in a drop of dimethyl sulfoxide and subsequently diluted with water. Thiopental, Org 20599 and Org 20549 were dissolved in water.

2.4. Data analysis

2.4.1. Description of the displacement curves

The sigmoid-E_{max} equation was fitted to the data in order to describe the displacement curves.

$$E_{drug} = \frac{E_{max}}{1 + \left[\frac{IC_{50}}{[drug]}\right]^{H}}. \quad (1)$$

In Eq. (1), [drug] is the concentration of test drug in mol/l. E_{max} is the experimentally determined binding of [^{3}H]TBOB in the absence of test drug, representing 100% binding. E_{drug} is the experimentally determined binding of [^{3}H]TBOB in the presence of the test drug and is expressed as a percentage of E_{max} binding. IC_{50} is the concentration of the test drug that produces 50% displacement of [^{3}H]TBOB and H is the Hill coefficient. IC_{50} and H were estimated by nonlinear regression analysis.

2.4.2. Isobolographic analysis of the data

The IC_{50} parameters obtained by the sigmoid-E_{max} model were plotted in an isobologram (Tallarida, 1992). In an isobologram, the concentration of one drug (e.g., GABA) is represented on the abscissa and the concentration of the other drug (the anaesthetic) is represented on the ordinate.

Each plotted point in the graph represents a pair of concentrations of the two drugs that reach the IC_{50} when added in combination. The straight line that connects the two plotted points of the pure single drugs is the isobolographic line. If

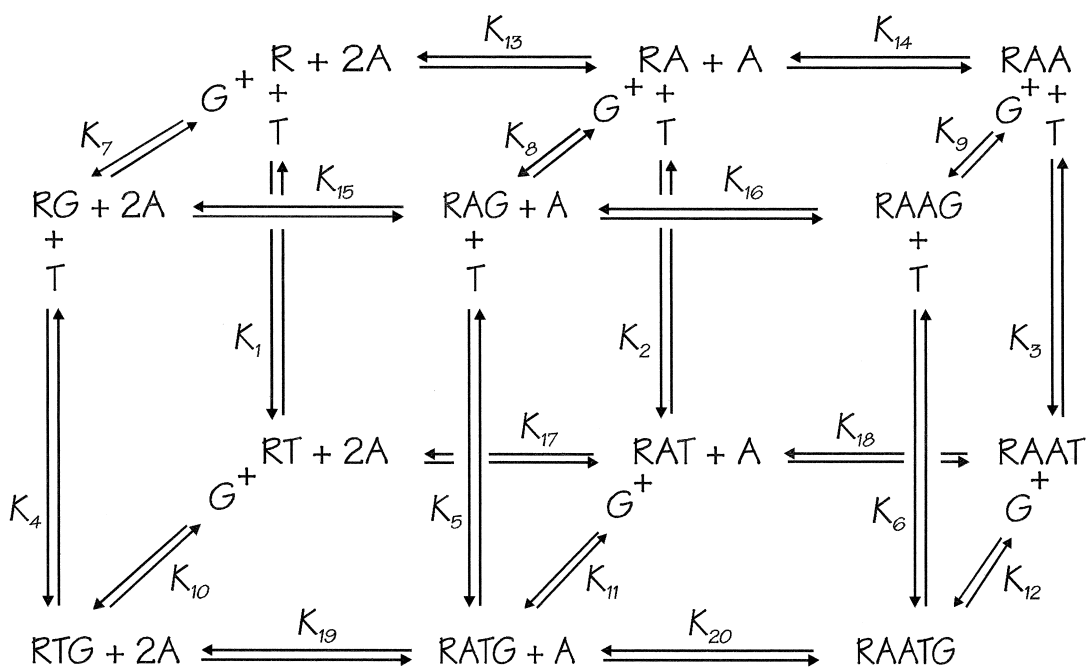

Fig. 1. The allosteric three-ligand molecular model for interactions between GABA, anaesthetics and [^{3}H]TBOB. The model reflects a set of bimolecular reversible reactions, in which R is a receptor site. Four receptor sites are assumed for three ligands: one site for GABA [G], two sites for the anaesthetic [A] and one site for [^{3}H]TBOB [T]. K_n's are dissociation constants. All four sites are allosterically coupled. GABA displaces [^{3}H]TBOB, so $K_4 > K_1$. The anaesthetic displaces [^{3}H]TBOB, so $K_2 > K_1$. GABA enhances the binding of the anaesthetic: $K_8 < K_7$. The two anaesthetic sites are positively coupled: $K_{14} < K_{13}$.

experimentally determined, data points lie on this straight line, then the drug effects are additive (no interaction). If the points lie below this line, then there is supraadditivity (synergy); if they lie above this line, then there is subadditivity (antagonism).

2.4.3. Molecular modelling of the data

A molecular model for allosteric interactions between GABA, an anaesthetic and [^{3}H]TBOB is depicted in Fig. 1. The model reflects a set of reversible bimolecular reactions, in which R is a receptor site. Three receptor sites are assumed: one site for GABA [G], a double one for the anaesthetic [A] and one for [^{3}H]TBOB [T]. The empty receptor complex is abbreviated as R. All reactions reached equilibrium before the incubations ended (Kenakin, 1997).

The model describes that GABA displaces, allosterically, [^{3}H]TBOB, thus, $K_4 > K_1$. Also the anaesthetic displaces allosterically [^{3}H]TBOB, thus $K_2 > K_1$. Since the displacement curves of the anaesthetics on the binding of [^{3}H]TBOB have a Hill coefficient factor greater than one, a double site for an anaesthetic must be assumed: K_{13} and K_{14}. Contrary to the negative effect of both GABA and the anaesthetic on the binding of [^{3}H]TBOB, GABA enhances allosterically the binding of an anaesthetic and vice versa, thus, $K_8 < K_7$. We can measure this enhancement, indirectly, through the effect of a combination of GABA and an anaesthetic on the binding of [^{3}H]TBOB. The factor K_8/K_7 shows the strength of the interaction between GABA and an anaesthetic.

2.4.4. Derivation of the equation describing the allosteric three-ligand molecular model

The molecular model is described with expressions for the dissociation constants (K_ds) of the drug-receptor complexes:

$$K_1 = \frac{[R]\cdot[T]}{[RT]} \qquad K_2 = \frac{[RA]\cdot[T]}{[RAT]} \qquad K_3 = \frac{[RAA]\cdot[T]}{[RAAT]}$$

$$K_4 = \frac{[RG]\cdot[T]}{[RTG]} \qquad K_5 = \frac{[RAG]\cdot[T]}{[RATG]} \qquad K_6 = \frac{[RAAG]\cdot[T]}{[RAATG]}$$

$$K_7 = \frac{[R]\cdot[G]}{[RG]} \qquad K_8 = \frac{[RA]\cdot[G]}{[RAG]} \qquad K_9 = \frac{[RAA]\cdot[G]}{[RAAG]}$$

$$K_{10} = \frac{[RT]\cdot[G]}{[RTG]} \qquad K_{11} = \frac{[RAT]\cdot[G]}{[RATG]} \qquad K_{12} = \frac{[RAAT]\cdot[G]}{[RAATG]}$$

$$K_{13} = \frac{[R]\cdot[A]}{[RA]} \qquad K_{14} = \frac{[RA]\cdot[A]}{[RAA]} \qquad K_{15} = \frac{[RG]\cdot[A]}{[RAG]} \qquad K_{16} = \frac{[RAG]\cdot[A]}{[RAAG]}$$

$$K_{17} = \frac{[RT]\cdot[A]}{[RAT]} \qquad K_{18} = \frac{[RAT]\cdot[A]}{[RAAT]} \qquad K_{19} = \frac{[RTG]\cdot[A]}{[RATG]} \qquad K_{20} = \frac{[RATG]\cdot[A]}{[RAATG]}.$$

These K_d values are dependent, in sets of four:

$K_1 \cdot K_{10} = K_7 \cdot K_4$
$K_2 \cdot K_{11} = K_8 \cdot K_5$
$K_3 \cdot K_{12} = K_9 \cdot K_6$
$K_1 \cdot K_{17} = K_{13} \cdot K_2$
$K_2 \cdot K_{18} = K_{14} \cdot K_3$
$K_4 \cdot K_{19} = K_{15} \cdot K_5$
$K_5 \cdot K_{20} = K_{16} \cdot K_6$
$K_7 \cdot K_{15} = K_{13} \cdot K_8$
$K_8 \cdot K_{16} = K_{14} \cdot K_9$
$K_{10} \cdot K_{19} = K_{17} \cdot K_{11}$
$K_{11} \cdot K_{20} = K_{18} \cdot K_{12}$

To describe the allosteric interactions, the following factors are introduced:

$K_{14} = \alpha K_{13}$
$K_2 = \beta K_1$
$K_8 = \gamma K_7$
$K_4 = \delta K_1$
$K_5 = \varepsilon K_4 = \varepsilon\delta K_1$

So:

α	Describes the change in K_d of the anaesthetic by binding of one anaesthetic molecule
β	Describes the change in K_d of TBOB by binding of one anaesthetic molecule
γ	Describes the change in K_d of GABA by binding of one anaesthetic molecule
δ	Describes the change in K_d of TBOB by binding of a GABA molecule
ε	Describes the change in K_d of TBOB by binding of both a GABA molecule and one anaesthetic molecule

To reduce the number of parameters to be estimated, we assumed that the effect of the second anaesthetic bound is equal to that of the first anaesthetic bound, thus:

$K_3 = \beta^2 K_1$

$K_6 = \varepsilon^2 K_4 = \varepsilon^2 \delta K_1$

$K_9 = \gamma^2 K_7$

The concentration of [^{3}H]TBOB bound is:

$$[\text{Bound}_{\text{tot}}] = [\text{RT}] + [\text{RAT}] + [\text{RAAT}] + [\text{RTG}] + [\text{RATG}] + [\text{RAATG}].$$

The total receptor concentration is the sum of free receptors and occupied receptors:

$$[\text{R}_{\text{tot}}] = [\text{R}] + [\text{RA}] + [\text{RAA}] + [\text{RG}] + [\text{RAG}] + [\text{RAAG}] + [\text{RT}] + [\text{RAT}] + [\text{RAAT}] + [\text{RTG}] + [\text{RATG}] + [\text{RAATG}].$$

The displacement curves are described in terms of fractional occupancy:

$$\frac{[\text{Bound}_{\text{tot}}]}{[\text{R}_{\text{tot}}]}.$$

Rearrangement of the above equations yields:

$$[\text{Bound}_{\text{tot}}] = \frac{[\text{R}]\cdot[\text{T}]}{K_1}\left(1 + \frac{[\text{A}]}{\beta K_{13}} + \frac{[\text{A}]^2}{\alpha\beta^2 K_{13}^2} + \frac{[\text{G}]}{\delta K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma\delta\varepsilon K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2\delta\varepsilon^2 K_7 K_{13}}\right),$$

$$[\text{R}_{\text{tot}}] = [\text{R}]\left\{1 + \frac{[\text{A}]}{K_{13}} + \frac{[\text{A}]^2}{\alpha K_{13}^2} + \frac{[\text{G}]}{K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2 K_7 K_{13}^2} + \frac{[\text{T}]}{K_1}\left(1 + \frac{[\text{A}]}{\beta K_{13}} + \frac{[\text{A}]^2}{\alpha\beta^2 K_{13}^2} + \frac{[\text{G}]}{\delta K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma\delta\varepsilon K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2\delta\varepsilon^2 K_7 K_{13}}\right)\right\},$$

In our experiments, $[T]/K_1 = 1$ because [T] is the concentration of [^{3}H]TBOB added, being 8 nM, and the K_d([^{3}H]TBOB) = 8 nM (van Rijn et al., 1990).

Thus, the overall expression describing the molecular model labelled with [^{3}H]TBOB is:

$$\frac{[\text{Bound}_{\text{tot}}]}{[\text{R}_{\text{tot}}]} = \frac{\left(1 + \frac{[\text{A}]}{\beta K_{13}} + \frac{[\text{A}]^2}{\alpha\beta^2 K_{13}^2} + \frac{[\text{G}]}{\delta K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma\delta\varepsilon K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma\delta\varepsilon^2 K_7 K_{13}}\right)}{1 + \frac{[\text{A}]}{K_{13}} + \frac{[\text{A}]^2}{\alpha K_{13}^2} + \frac{[\text{G}]}{K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2 K_7 K_{13}^2} + \left(1 + \frac{[\text{A}]}{\beta K_{13}} + \frac{[\text{A}]^2}{\alpha\beta^2 K_{13}^2} + \frac{[\text{G}]}{\delta K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma\delta\varepsilon K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2\delta\varepsilon^2 K_7 K_{13}}\right)}. \quad (2)$$

The equation was fitted to the data using nonlinear regression analysis. The parameters K_7, K_{13}, α, β, γ, δ and ε were estimated. [G], the concentration of GABA, and [A], the concentration of the anaesthetic, are known.

2.5. Statistical analysis

F-tests were used to test whether the model with additional parameters fitted the data better than the model with fewer parameters. Parameter estimates were tested for differences with analysis of variance (ANOVA). We used the program Prism 2.0 (GraphPad Software) for both the nonlinear regression analysis and the statistical analysis.

3. Results

To test whether endogenous GABA was removed by the three freezing-washing cycles, we added bicuculline-methochloride (20 μM) to the samples. Bicuculline-methochloride enhanced the binding of [^{3}H]TBOB by only 6 ± 1%

(mean ± S.E.M., $n = 27$), indicating that hardly any GABA was present (Squires and Saederup, 1987; van Rijn et al., 1992).

3.1. Fitting the sigmoid-E_{max} Eq. (1) to the data

GABA displaced [^{3}H]TBOB with an IC_{50} of 2.48 ± 0.26 μM (mean ± S.E.M., $n = 4 \times$ (three to six in triplicate)). The Hill coefficient, 0.92 ± 0.02, was not significantly different from unity. The displacement curves of GABA were determined in pairs together with the curves for the anaesthetics, so that each anaesthetic had its own reference GABA data. The IC_{50} of GABA in the thiopental experiment was 2.99 μM, in the propofol experiment 2.96 μM, in the Org 20599 experiment 2.19 μM, and in the Org 20549 experiment 1.76 μM. In none of the experiments did the Hill coefficient differ from unity.

The four tested anaesthetics displaced [^{3}H]TBOB both in the absence and in the presence of GABA. Parameter estimates (IC_{50} and H) are given in Table 1; data points and best fits are given in Fig. 2. The magnitudes of the IC_{50}s of the anaesthetics were lower at higher concentrations of GABA. Two-way ANOVA of the drug × GABA concentration interaction indicated for both thiopental and propofol a main effect for GABA concentration with $F(4,37) = 240$; $P < 0.0001$ and for both Org compounds a main effect for GABA concentration with $F(3,24) = 9.0$; $P < 0.001$. The magnitudes of the Hill coefficients of thiopental and propofol were lower at higher concentrations of GABA. Two-way ANOVA of the drug × GABA concentration interaction showed a main effect for GABA concentration with $F(4,37) = 8.6$, $P < 0.0001$. The Hill coefficients of Org 20599 and Org 20549 did not depend on the GABA concentration and were not different from unity.

3.2. Isobolographic analysis

Isobolograms were made for 50% displacement of [^{3}H]TBOB (Fig. 3). Parameter estimates of the anaesthetics in the presence of 1 μM GABA were compared to the theoretical additive values using 95% confidence intervals.

All experimentally determined data points of combinations of GABA and an anaesthetic were below the isobolographic line. There was no overlap between the 95% confidence intervals of the theoretical additive concentrations (the straight line

Table 1
Parameter estimates of the sigmoid-E_{max} curve fitted to the experimental data

GABA [μM]	IC_{50} [μM]	Hill	*n*
Thiopental			
0	86.8 ± 1.7	2.40 ± 0.11	6
0.4	53.7 ± 3.5	1.80 ± 0.21	5
1	40.9 ± 2.4	1.76 ± 0.15	5
4	24.1 ± 1.6	1.65 ± 0.15	4
8	18.6 ± 3.3	1.47 ± 0.23	4
Propofol			
0	37.6 ± 0.9	2.16 ± 0.09	5
0.4	20.8 ± 0.5	1.80 ± 0.07	4
1	12.9 ± 0.6	1.54 ± 0.09	6
4	6.81 ± 0.8	1.38 ± 0.13	4
8	3.67 ± 0.3	1.52 ± 0.19	4
Org 20599			
0	1.08 ± 0.11	0.89 ± 0.11	6
0.4	0.523 ± 0.026	1.26 ± 0.08	3
1	0.361 ± 0.039	1.23 ± 0.11	4
4	0.212 ± 0.020	1.18 ± 0.10	4
Org 20549			
0	53.6 ± 4.6	0.91 ± 0.07	5
0.4	8.22 ± 0.91	0.86 ± 0.07	3
1	4.84 ± 0.47	0.86 ± 0.06	4
4	2.00 ± 0.63	0.73 ± 0.16	3

The anaesthetics displaced [^{3}H]TBOB. The sigmoid-E_{max} Eq. (1) was fitted to the experimental data. The parameter estimates and the S.E. of fit are given. *N* is the number of experiments in triplicate. The IC_{50} of the anaesthetics decreased with increasing concentrations of GABA (ANOVA, $P < 0.001$). The Hill coefficients of thiopental and propofol decreased with increasing concentrations of GABA (ANOVA, $P < 0.001$). The Hill coefficients of Org 20599 and of Org 20549 were not different from unity.

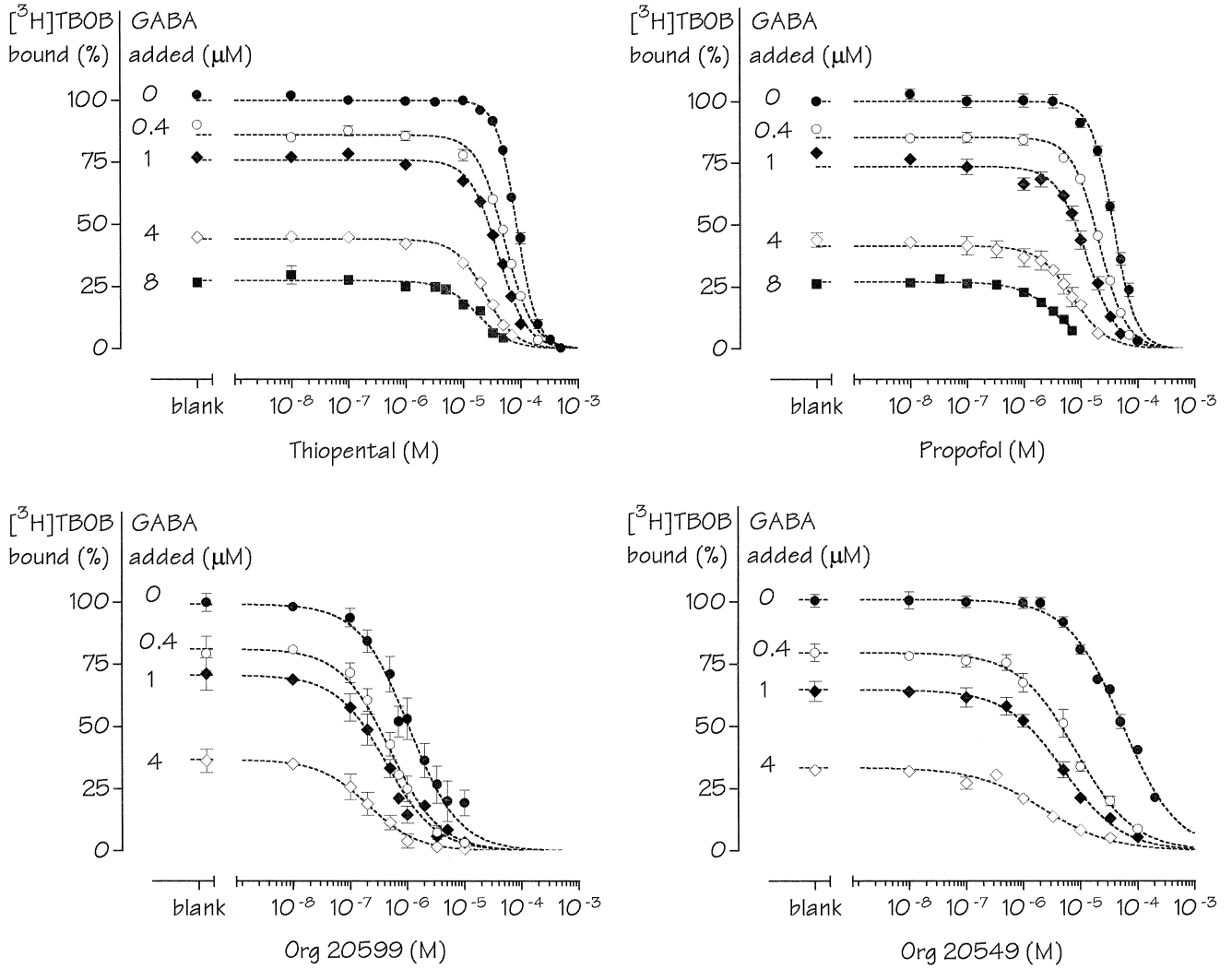

Fig. 2. The effect of the anaesthetics on the binding of [^{3}H]TBOB (8 nM) in the presence of several concentrations of GABA (data points: mean ± S.E.M., $n = 3$–6 in triplicate). The anaesthetics displace [^{3}H]TBOB. The sigmoid-E_{max} model, Eq. (1), is fitted to the data (dotted lines). Parameter estimates are given in Table 1.

which connects the two points of the pure drugs on the axes) and the actually measured concentrations, so the isobolograms showed that all four anaesthetics interacted synergically with 1 μM GABA in displacing 50% [^{3}H]TBOB.

3.3. Model fitting

The general equation for the amount of bound [^{3}H]TBOB (Eq. (2)) seems rather complex. However, when fitting the model to the data, it appeared that the factors β and δ must be large in order to achieve complete displacement of [^{3}H]TBOB binding. *F*-tests on the fit results of the data of the single compounds showed that β and δ could be fixed at high values (i.e., 1000), so the model can be simplified to that shown in Fig. 4 and Eq. (2) can be simplified to:

$$\frac{[\text{Bound}_{tot}]}{[\text{R}_{tot}]} = \frac{1}{1 + \frac{[\text{A}]}{K_{13}} + \frac{[\text{A}]^2}{\alpha K_{13}^2} + \frac{[\text{G}]}{K_7} + \frac{[\text{A}]\cdot[\text{G}]}{\gamma K_7 K_{13}} + \frac{[\text{A}]^2\cdot[\text{G}]}{\alpha\gamma^2 K_7 K_{13}^2} + 1}. \quad (3)$$

This leaves only the following parameters to be estimated: K_7, K_{13}, α and γ.

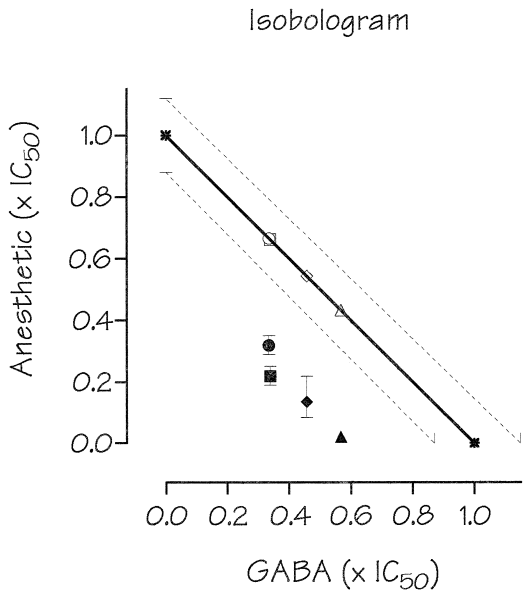

Fig. 3. Isobologram of combinations of 1 μM GABA with anaesthetics. Abscissa: concentration of GABA in fractions of its IC_{50}; ordinate: concentration of the anaesthetic in fractions of its IC_{50}. The asterisks plotted on the axis represent the IC_{50} values of the pure compounds, defining the isobolographic line. Open symbols represent the theoretical additive concentrations of the anaesthetics needed to displace 50% [^{3}H]TBOB binding in the presence of 1 μM GABA. Closed symbols represent observed concentrations: ●, thiopental; ■, propofol; ◆, Org 20599; ▲, Org 20549. Dotted lines indicate the 95% confidence intervals. The isobologram demonstrates synergy between GABA and these anaesthetics.

Eq. (3) was first fitted to the data for the pure drugs. In the presence of GABA only, [A] = 0 and Eq. (3) simplifies further to:

$$\frac{[\text{Bound}_{\text{tot}}]}{[\text{R}_{\text{tot}}]} = \frac{1}{1 + \frac{[\text{G}]}{K_7} + 1}. \tag{4}$$

K_7 is the dissociation constant of GABA (Table 2), which describes the strength of the binding of GABA to the complex.

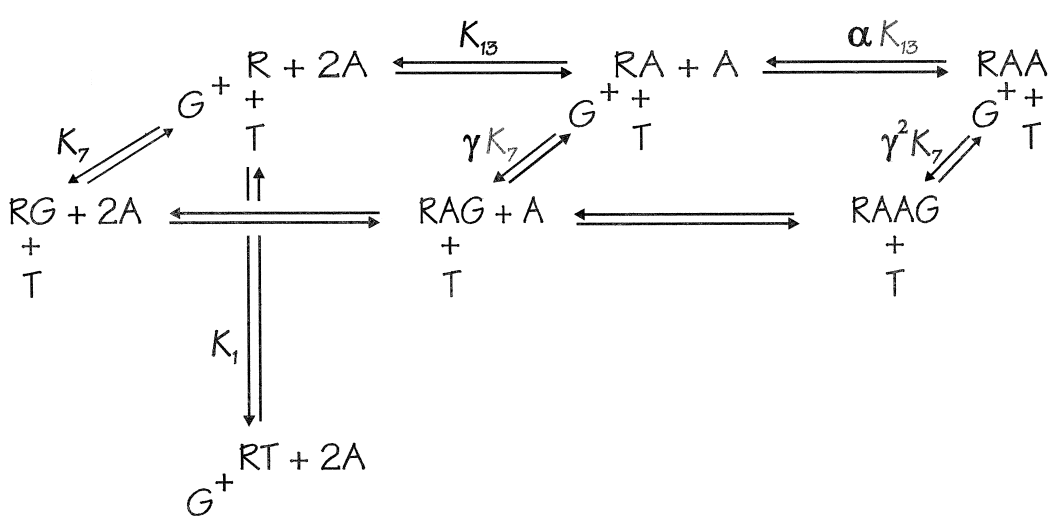

Fig. 4. The allosteric three-ligand molecular model with parameters β, δ and ε in Eq. (2) fixed at 1000 (see Section 2). Parameter γ quantifies the coupling between GABA and the anaesthetic, and α quantifies the coupling between the anaesthetic sites. Parameter estimates are given in Table 2 and in Table 3. Curves are given in Fig. 5.

Table 2
Parameter estimates of the allosteric three-ligand molecular model fitted to the experimental data for the pure drugs

Drug	K_d (K_7 or K_{13}) [μM]	# Sites	α
GABA	1.25 ± 0.07	1	–
Thiopental	210 ± 10	2	0.105 ± 0.013
Propofol	92.0 ± 4.8	2	0.092 ± 0.011
Org 20599	0.489 ± 0.033	1	–
Org 20549	26.4 ± 1.1	1	–

Experimental data of the pure drugs (GABA, or anaesthetic in the absence of GABA) were fitted to the allosteric three-ligand molecular model, which reduces to Eq. (4) (GABA) or to Eq. (5) (anaesthetics), (see Section 3). The parameter α quantifies the coupling between the two anaesthetic sites. Best-fit values and S.E. of fit are given. For GABA and for the Org compounds, best fits were obtained with one binding site.

In the presence of an anaesthetic only, $[G] = 0$ and Eq. (3) simplifies to:

$$\frac{[\text{Bound}_{tot}]}{[R_{tot}]} = \frac{1}{1 + \frac{[A]}{K_{13}} + \frac{[A]^2}{\alpha K_{13}^2} + 1}. \quad (5)$$

K_{13} is the dissociation constant of the anaesthetics (Table 2), which describes the strength of the binding of the anaesthetics to the complex. Parameter α describes the strength of the allosteric interaction between two binding sites for an anaesthetic.

For GABA, as well as for the Org compounds, best fits were obtained with a one-binding site model (i.e., α is large, ≥ 1000). For propofol, an allosteric two-binding site model fitted best, such that after the first molecule has bound, the second molecule bound with 10-fold greater affinity than the first ($\alpha \pm 0.1$). The same applied to thiopental. All estimates of the parameters are given in Table 2.

Next, Eq. (3) was fitted to the data for the combinations of drugs, using the parameter estimates in Table 2, leaving only γ to be fitted (the strength of the interaction between GABA and the anaesthetic). Table 3 shows the parameter estimates of

Table 3
Parameter estimates of γ

GABA [μM]	γ
Thiopental	
0.4	0.369 ± 0.013
1	0.391 ± 0.015
4	0.336 ± 0.014
8	0.270 ± 0.021
Propofol	
0.4	0.324 ± 0.015
1	0.268 ± 0.015
4	0.201 ± 0.010
8	0.125 ± 0.007
Org 20599	
0.4	0.223 ± 0.030
1	0.399 ± 0.061
4	0.298 ± 0.021
Org 20549	
0.4	0.070 ± 0.008
1	0.083 ± 0.007
4	0.077 ± 0.017

The allosteric three-ligand molecular model was fitted to the experimental data for the anaesthetics in the presence of GABA. The allosteric three-ligand molecular model (see Eq. (3) in Section 3) was fitted to the experimental data for the combinations of drugs using the parameter estimates of the pure drugs given in Table 2. Parameter γ quantifies the coupling between GABA and the anaesthetic. GABA enhances the affinity of the anaesthetic and vice versa with a factor $1/\gamma$. Best-fit values and S.E. of fit are given.

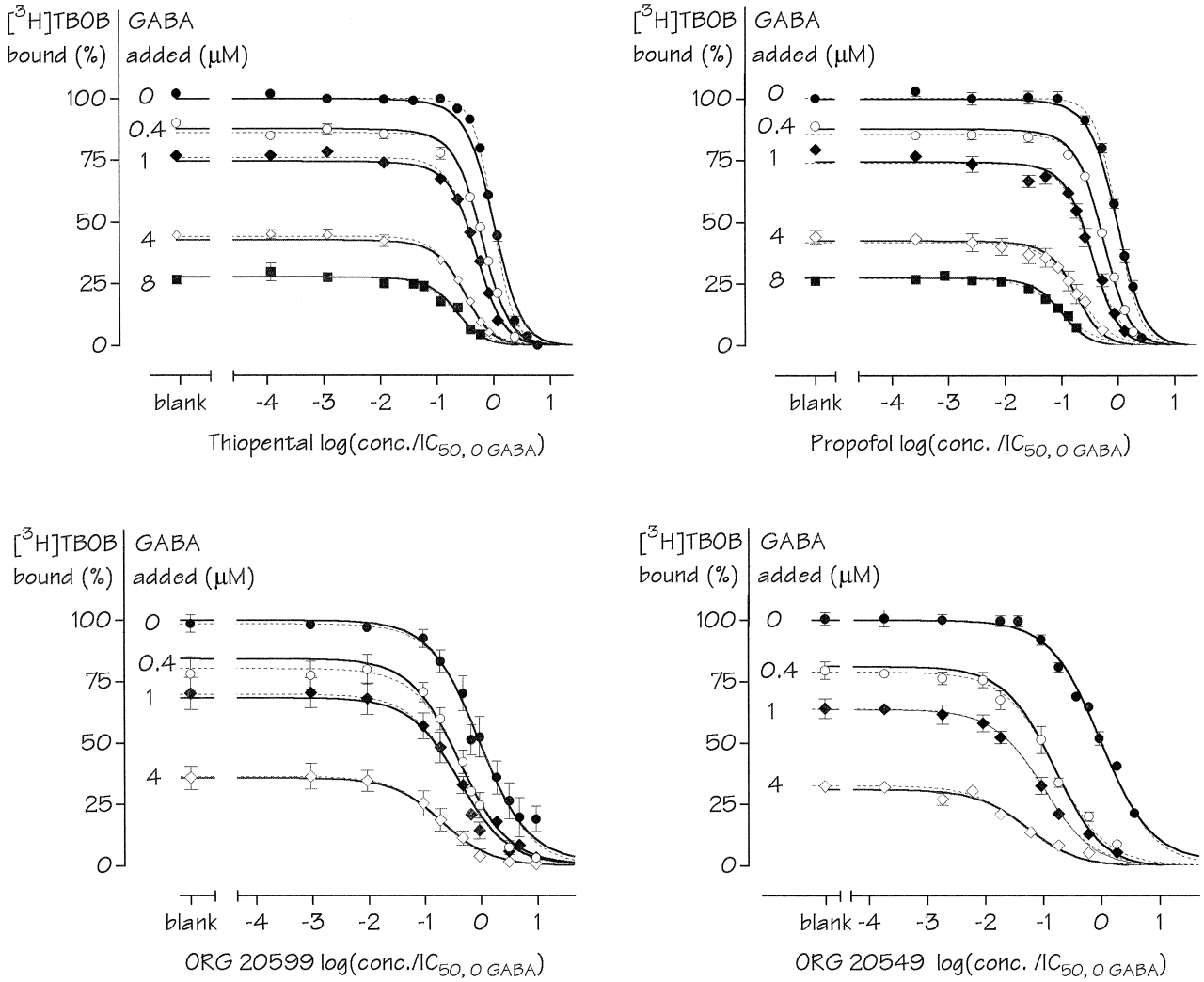

Fig. 5. The effect of the anaesthetics on the binding of [^{3}H]TBOB (8 nM) in the presence of several concentrations of GABA (data points: mean ± S.E.M., $n = 3–6$ in triplicate. The allosteric three-ligand model is fitted to the data (solid lines). Dotted lines indicate the best sigmoid-E_{max} model. Parameter estimates of the model are given in Table 2 and in Table 3. The IC_{50} of an anaesthetic in the absence of GABA was normalised. This graph shows that the allosteric three-ligand model fits well to the experimental data.

γ. Fig. 5 shows the experimental data together with the curves fitted according to the allosteric three-ligand molecular model.

4. Discussion

In this study, we investigated the influence of GABA on the displacement of [^{3}H]TBOB by four different anaesthetics. Our data confirm earlier reports that: (1) GABA and various anaesthetics displace ligands bound to the convulsant site of the $GABA_A$ receptor complex (Concas et al., 1994), and (2) GABA shifts the displacement curves of these anaesthetics to the left (Johnston and Willow, 1982; Harris and Allan, 1985; Maksay and Ticku, 1988; Maksay et al., 1991). Based on these well-known findings, we raised two questions. The first question is whether the effect of a combination of GABA and an anaesthetic is greater than, equal to, or less than simple addition of the individual effects (called synergy (or superadditivity), additivity and antagonism (or subadditivity), respectively) (Berenbaum, 1989). The second question is which mechanism underlies the observed interaction.

To answer the first question, we used the isobole method (Tallarida, 1992). We made isobolograms for 50% displacement of [^{3}H]TBOB for the experiments with 1 μM GABA. Fig. 3 shows that for all four anaesthetics the required dose to displace 50% [^{3}H]TBOB was less than the theoretical additive dose. Therefore the interaction between 1 μM GABA and each of the tested anaesthetics was synergic, which answers the first question.

The isobole method, however, is of limited use for analysing drug interactions. In the first place, the method does not allow extrapolation from one dose combination to other dose combinations. Neither is the method convenient for extrapolation to other magnitudes of effect, which would only be allowed if all concentration-effect curves had the same Hill coefficient (i.e., the curves were parallel). Since this was not the case in the experiments presented here (see Table 1), we had to make a new isobologram for each set of parameters.

The second, more important, reason that the isobole method is of limited use is that it is an empirical method and therefore not suitable to answer our second question: which mechanism underlies the observed interaction? Physiological molecular models are needed to answer this. Berenbaum (1989) opposed considering the mechanism of action of drugs when describing drug interactions. We think that observed interactions should be used to construct possible mechanisms of action. Molecular models have been applied extensively to a variety of receptor classes (Birdsall et al., 1978; Jakubik et al., 1997; Kenakin, 1997; Leff et al., 1997). However, with respect to $GABA_A$ receptors, molecular models have been mainly applied to interactions between two ligands: benzodiazepines and GABA (Ehlert et al., 1983). We designed a molecular model and tested whether it could describe our data.

The molecular model consists of four separate sites for three different ligands: one site for GABA, two sites for the anaesthetics and one site for [^{3}H]TBOB. The two sites for the anaesthetics are needed in view of the high Hill coefficients found for thiopental and propofol (Hill Venning et al., 1996). All four sites are allosterically coupled (Wong et al., 1984; Sieghart, 1992; Sivilotti and Nistri, 1991) in such a way that the binding of any one drug changes the affinity of the binding of the other drugs (Birdsall et al., 1978; Burgen, 1981). This allosterical three-ligand molecular model is fully described by Eq. (2). This model encompasses simpler models described earlier (Ehlert, 1988, Jakubik et al., 1997). Ehlert described the allosteric interaction between two ligands. This two-ligand model corresponds to the leftmost lateral face of the double cube in Fig. 1. Jakubik et al. described the allosteric interactions between three ligands, two of which are competitive. This model corresponds to the entire left cube in Fig. 1, provided that the $K_4 \gg K_1$.

The interaction between GABA and the anaesthetics was measured indirectly through their combined influence on the binding of the radiolabeled ligand [^{3}H]TBOB, which was used as a tracer ligand. The allosteric three-ligand model presented in Fig. 1 described the experimental data adequately with respect to the IC_{50}s (Eq. (2) reduced to Eq. (3), values of γ in Table 3, results in Table 4, dose–response curves in Fig. 5). However, the GABA dependency of the Hill coefficient of thiopental and propofol does not show up in the model. This deficiency might be due to the fact that the model does not take into account the heterogeneity of the $GABA_A$ receptor complex (Fritschy and Möhler, 1995). The coupling between GABA and the anaesthetics might depend on the subunit composition of the receptors (Sanna et al., 1995; Jakubik et al., 1997). It will be a challenge to repeat our studies with various subtypes of the receptor complex as soon as subtype-selective drugs are available (Ruano et al., 1992). In addition, the model presumably is still too simple: multiple, allosterically coupled, GABA binding sites may be present on one receptor complex. The concerted action of two GABA molecules and two anaesthetic molecules in displacing the tracer ligand [^{3}H]TBOB might explain the GABA dependency of the Hill coefficient. To test this latter hypothesis, the model in Fig. 1 must be augmented with a second GABA site, which would greatly increase the number of parameters to be estimated.

To reduce the number of parameter estimates, we made the further assumption that the interaction factor between the binding of two anaesthetic molecules, and one GABA molecule is equal to the square of the interaction factor between one anaesthetic molecule and GABA ($K_9 = \gamma^2 K_7$). In order to test whether this assumption is valid, we increased, by interpolation, the number of data points. When in the allosterical three-ligand model $K_9 = \gamma^x K_7$ and the model was fitted to the extended data set, x was indeed nearly 2, to be exact 1.8, justifying the assumption of $K_9 = \gamma^2 K_7$. This assumption applied to thiopental and propofol only, and not to the Org compounds, because a single binding-site suffices to describe the data for the Org compounds.

To show that the allosterical three-ligand model is the simplest physiological model that can describe the data, we evaluated two simpler models as well. The first simple model assumed a competitive interaction between GABA and the anaesthetics. This model is described by Eq. (3) if γ is very large (i.e., $\gamma > 1000$). The rationale behind this model is that anaesthetics might behave like GABA notwithstanding their separate binding sites. It has been suggested, for example, that benzodiazepines enhance the effect of GABA without directly influencing the Cl^- channel (Haefely, 1990). Addition of benzodiazepines should then have the same effect as addition of GABA (Macdonald and Olsen, 1994). The IC_{50}s of data simulated according to this competitive model showed however that this model did not adequately describe our experimental results (Table 4 and Fig. 6). The second simple model assumes separate but independent binding sites for GABA and the anaesthetic (Kenakin, 1997). This model is described by Eq. (3) if $\gamma = 1$. The IC_{50}s of data simulated using this model did not agree with the experimentally obtained IC_{50}s either (Table 4 and Fig. 6). This result shows that allosterical coupling between the binding sites is essential to describe the experimental data.

A few words about the clinical implications of our study. Knowledge about interactions, quantified over the full effective dose-range, can help clinicians to optimise their therapeutic strategy. They might choose drugs that show minimal interactions with endogenous compounds in order to limit the individual variation of the effects, or they can choose

Table 4
Normalised IC_{50} values of the sigmoid-E_{max} Eq. (1) for the experimental data and for the three molecular models

GABA [μM]	Simple model 1		Simple model 2		Proposed model		Experiment	
	One common site		Separate independent sites		Allosteric three-ligand model		Measured IC_{50}	
	IC_{50}	Hill	IC_{50}	Hill	IC_{50}	Hill	IC_{50}	Hill
Thiopental								
0	1.00	1.8	1.00	1.8	1.00	1.8	1.00	2.4
0.4	1.08	1.8	0.94	1.8	0.66	1.8	0.62	1.8
1	1.18	1.8	0.88	1.8	0.46	1.8	0.47	1.8
4	1.63	1.8	0.77	1.7	0.32	1.8	0.28	1.7
8	2.09	1.9	0.73	1.7	0.24	1.7	0.21	1.5
Propofol								
0	1.00	1.8	1.00	1.8	1.00	1.8	1.00	2.2
0.4	1.08	1.8	0.94	1.8	0.66	1.8	0.55	1.8
1	1.18	1.8	0.88	1.8	0.46	1.8	0.34	1.5
4	1.63	1.8	0.77	1.7	0.32	1.8	0.18	1.4
8	2.09	1.9	0.73	1.7	0.24	1.7	0.10	1.5
Org 20599								
0	1.00	1.0	1.00	1.0	1.00	1.0	1.00	0.9
0.4	1.25	1.0	0.87	1.0	0.52	1.0	0.49	1.3
1	1.65	0.9	0.75	1.0	0.35	1.0	0.34	1.2
4	3.82	0.9	0.60	1.0	0.21	1.0	0.20	1.1
Org 20549								
0	1.00	1.0	1.00	1.0	1.00	1.0	1.00	0.9
0.4	1.25	1.0	0.87	1.0	0.17	1.0	0.15	0.9
1	1.65	0.9	0.75	1.0	0.09	1.0	0.09	0.9
4	3.82	0.9	0.60	1.0	0.05	1.0	0.04	0.7

GABA dependency of the IC_{50} values for the three molecular models. The last column gives normalised experimental parameter estimates. The GABA dependency of the IC_{50} values of the two simple models differs from the experimental results (two-way ANOVA: thiopental and propofol: $F(4,38) > 23$, $P < 0.001$; Org compounds: $F(3,22) > 14$, $P < 0.001$). The GABA dependency of the IC_{50} values of the three-ligand allosteric model is not significantly different from the experimental results (two-way ANOVA: thiopental and propofol: $F(4,38) < 0.32$, $P > 0.86$; Org compounds: $F(3.33) < 0.02$, $P > 0.96$. The Hill coefficients of none of the models showed GABA dependency. The data of this table are given in Fig. 6.

powerful combinations of drugs with maximal interaction in order to minimise drug load and thereby side effects (Deckers et al., 1997). Clinicians can also benefit from knowledge about the drug dependency of the slope factor of the dose–effect

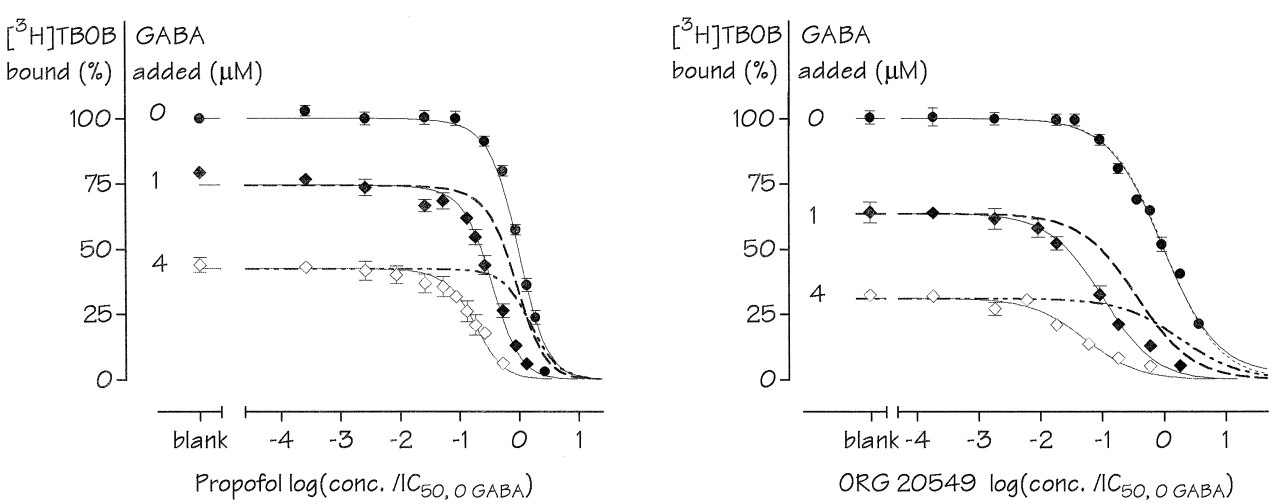

Fig. 6. Curves of the three molecular models for propofol and for Org 20549 in the presence of two concentrations of GABA. Parameter values for all combinations tested are given in Table 4. The IC_{50} of an anaesthetic in the absence of GABA was normalised. The solid lines indicate the allosteric three-ligand molecular model Eq. (3). The dotted-and-dashed line is the curve of simple model 1 (one common site), shown for 1 μM GABA, the dashed line is the curve of simple model 2 (separate independent sites), shown for 4 μM GABA. This graph illustrates that neither of the two simple models fits the experimental data.

curve. Slope factors determine the progression of effect as a function of the dose and therefore correlate with the controllability of the effects. This latter is relevant for the dose–effect curves of general anaesthetics, which have steep dose–response curves (Dirksen et al., 1990). The clinically important questions whether the four tested anaesthetics bind to the same site or to different sites, and whether there is mutual allosteric interaction, are the subject of ongoing studies.

In summary, in this paper we showed that the interaction between GABA and each of the anaesthetics in displacing [^{3}H]TBOB can be described adequately by a molecular model of an allosteric interaction between three ligands. This allosteric three-ligand model needs only one parameter γ to quantitatively describe the interaction between GABA and anaesthetics over the full effective dose range. A γ less than 1 indicates synergy. We could compare the strength of the synergic interaction between different compounds, since we could also calculate confidence intervals (Table 3). We showed that the interaction GABA-propofol was greater than the interaction GABA-thiopental and that the interaction GABA-Org 20549 was greater than the interaction GABA-Org 20599.

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