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Binary combinations of propofol and barbiturates on human α_1 glycine receptors expressed in *Xenopus* oocytes

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Abstract

To test whether there is a common site of action for intravenous anaesthetics at the glycine receptor, the effects of binary combinations of thiopentone, pentobarbitone, methohexitone, and propofol have been tested on human α_1 glycine receptors expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp techniques. Thiopentone (5–40 μM), pentobarbitone (25–400 μM) and propofol (2–100 μM) (but not methohexitone), potentiated the glycine-induced (50 μM) current in a dose-dependent manner, with the maximum potentiation observed to be 218%, 400%, and 576%, respectively. In binary combination with thiopentone, pentobarbitone or propofol, methohexitone reduced potentiation compared to that by the individual anesthetics to 190%, 260% and 460%, respectively. Combination of thiopentone and pentobarbitone (50 μM) increased potentiation, compared to that by thiopentone alone. Binary combinations of propofol with either thiopentone or pentobarbitone showed more potentiation, compared to that observed with the individual anesthetics. Our results indicate that thiopentone, pentobarbitone and propofol all act as positive allosteric modulators at the α_1 glycine receptor. In contrast, methohexitone has no action alone but acts as a competitive antagonist to thiopentone, pentobarbitone and propofol. We suggest that, on the basis of these results, these four intravenous anaesthetics share a common site of action at the glycine receptor.

Keywords: Anaesthetic, intravenous; Barbiturate; Propofol; Glycine receptor

1. Introduction

General anaesthetics are among the most widely used and important therapeutic agents. Despite their wide use, their mechanism of action remains controversial. In contrast to most other classes of drugs which are assumed to act on specific protein receptors, anaesthetic action was often attributed to actions at multiple nonspecific sites. The lipid bilayer of neuronal membranes was long considered the primary target for general anaesthesia (Meyer, 1899; Overton, 1901). However, as a result of research over recent years, it now appears that their primary sites of action are neuronal proteins, especially the ligand-gated ion channels (Harris et al., 1995; Flood and Krawsowski, 2000; Yamakura et al., 2001).

The effect of anaesthetics on ion channels gated by GABA and glutamate has been the subject of numerous studies (Harris et al., 1995; Mihic and Harris, 1996). It has been demonstrated that the majority of intravenous anaesthetics, at clinically relevant concentrations, share the common feature of potentiation of the action of GABA at the $GABA_A$ receptor (Pistis et al., 1997; Patten et al., 2001).

However, particularly in the brain stem and spinal cord, the majority of inhibitory neuronal pathways use the neurotransmitter glycine and, it is now recognized, glycine receptors are widely distributed throughout the CNS (Snyder, 2000; Tao and Ye, 2002). Furthermore, recent studies have revealed that the majority of inhalational anaesthetics (Cheng and Kending, 2002) and some intravenous anaesthetics act as positive allosteric modulators of the strychnine-sensitive glycine receptor (Mascia et al., 1996; Downie et al., 1996; Daniels and Roberts, 1998).

In addition to asking at which receptors anaesthetics act, it is important to identify whether anaesthetics share a common site of action at a receptor protein, or whether distinct sites are targeted. One means of studying this question is to explore the effects of binary combinations of anaesthetics to test whether they behave in an additive manner, or not.

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We report on experiments using the *Xenopus laevis* oocyte expression system to study the effects of binary combinations of four intravenous anaesthetics on homomeric α_1 human glycine receptors. Three of the anaesthetics are structurally related (thiopentone, pentobarbitone and methohexitone) and one is structurally distinct (propofol).

2. Materials and methods

cDNA encoding the adult human α_1 glycine receptor subunit, inserted into the *Eco*RI site of pBluescript SK^-, was transformed using a heat-shock method into *E.coli*. Bluescript SK^-/α_1 DNA was prepared from overnight cultures of recombinant cells using a miniprep method based on alkaline lysis. It was then linearized with *Eco*RV (Wizard® Plus SV Minipreps DNA Purification System, Promega). Complete digestion produced a single band of apparent molecular mass 4.7 kDa on an ethidium bromide-stained agarose gel. cRNA was synthesized in vitro using the T3 bacteriophage promoter (mCAP mRNA capping kit, Stratagene).

The cRNA was microinjected using an hydraulic microinjector (50 nl at 1 μg μl^{-1}) into oocytes (stage IV/V) harvested from mature female *X. laevis*. The oocytes were kept in sterile pots containing modified Barth's solution (mM): NaCl 100, KCl 1, $NaHCO_3$ 2, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, HEPES 10, pH 7.4, supplemented with penicillin and streptomycin (100 μg μl^{-1} each).

Injected oocytes were defolliculated prior to making electrical recordings, either by treatment with 0.5 mg ml^{-1} collagenase A (Sigma) for 30 min (Daniels and Roberts, 1998) or manual removal of the vitelline envelope and associated follicular cells. In control experiments, no significant difference was observed between the effects of either propofol or pentobarbitone on currents evoked by 50 μM glycine from oocytes defolliculated using the two different methods (results not shown).

Current responses to bath applied glycine were recorded on a potentiometric chart recorder using the two-electrode voltage-clamp technique with a holding potential of −60 mV. During electrical recording, oocytes were perfused with frog Ringer: (mM) NaCl 120, KCl 2, $CaCl_2$ 10, HEPES 10, pH 7.4. Anaesthetics were applied to the oocytes dissolved in the Ringer. Pentobarbitone (Na-salt) and thiopentone (Na-salt) were purchased from Sigma. Methohexitone was obtained in a commercial formulation for intravenous use as a mixture of methohexitone sodium (500 mg) and sodium carbonate (30 mg) (Brietal Sodium; Eli Lilly). Propofol (Diprivan, Zeneca) was used as 1% w/v solution in Intralipid®, diluted into Ringer, as appropriate. Intralipid in Ringer, at the highest concentration used, had no effect on currents elicited by glycine.

Current measurements were made on oocytes 1–3 days after injection. The protocol to test the effect of anaesthetics on the current elicited by glycine was as follows: a control response to 50 μM glycine, dissolved in Ringers' solution and applied by bath perfusion for 30 s, was elicited; this was followed by perfusion with anaesthetic (in Ringer) for 30 s and then with glycine (50 μM) and anaesthetic together and finally a second control using 50 μM glycine alone was determined. For studies of the effect of two anaesthetics together, in a single oocyte, the effect of each anaesthetic alone was established followed by the effect of the binary combination of them, with a control response to 50 μM glycine between every application of anaesthetic and at the beginning and end of the complete sequence of test applications.

2.1. Statistical analysis

Current responses were measured by hand from the chart record as the peak amplitude. Current responses, for each oocyte, in the presence of anaesthetic were normalized with respect to the amplitude of the control response to 50 μM glycine for that oocyte and expressed as a percentage of the control response. Where appropriate, concentration–response curves were constructed using a standard logistic model to determine EC_{50} and Hill coefficients (h) (Origin 6, OriginLab).

$$\text{Response} = \frac{\min - \max}{1 + (x/\text{EC}_{50})^h} + \max$$

where: min = the minimum or initial response, max = the maximum or final response and x = concentration of glycine (or anaesthetic).

When fitting the data to the function, the minimum value was always fixed. Other parameters were fixed as dictated by the data (details given in the appropriate figure legends) and the best-fit established by minimising χ^2.

Further statistical analyses to test for differences in response were performed using one-way ANOVA and Student's t-test. Individual values throughout the paper are given as mean ± standard error of the mean.

3. Results

At a holding potential of −60 mV, oocytes pre-injected with cRNA encoding the human α_1 glycine receptor subunit responded to bath applied glycine (10–1000 μM) with a concentration-dependent inward current, with a calculated EC_{50} of 201 ± 9 μM and h (Hill coefficient) 2.6 ± 0.1 (Fig. 1). This value compares well with previous estimates of the EC_{50} for the action of glycine at the human homomeric α_1 receptor expressed in *Xenopus* oocytes, 190 ± 7 μM (Roberts et al., 1996) and 197 ± 4 μM (Daniels and Roberts, 1998). The Hill coefficient (2.6) indicates that at least two molecules of glycine are required to activate the receptor, again consistent with the previous reports (Roberts et al., 1996).

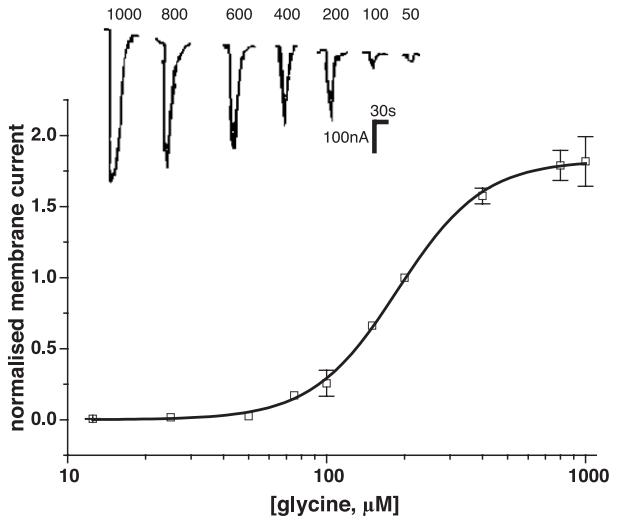

Fig. 1. Glycine concentration–response curve established from data obtained from oocytes expressing homomeric α_1 glycine receptors. Inward currents were measured in oocytes voltage-clamped at −60 mV in response to bath applied glycine (see insert, glycine concentrations given in μM and applied for 20 s). The points (□) represent the mean current response, normalized with respect to the current elicited by 200 μM glycine, from eight independent experiments ($n = 8$). The error bars indicate standard error of the mean at each data point. The line represents the best fit to a logistic function (see text) with $EC_{50} = 201 \pm 9$, $h = 2.6 \pm 0.1$, min = 0 (fixed), max = 1.8 ± 0.1.

Anaesthetics potentiate the response of the glycine receptor at low agonist concentrations (approximately EC_5), whereas higher concentrations (>EC_{50}) negatively modulate receptor function (Daniels and Roberts, 1998). We therefore examined the anaesthetic effects on currents elicited by 50 μM glycine, which we established represented a concentration approximately equivalent to the EC_5 (Fig. 1).

3.1. Anaesthetic potentiation

Pentobarbitone (25–400 μM) and thiopentone (5–40 μM) potentiated the current evoked by 50 μM glycine (EC_5), with a maximum observed potentiation of 400% (EC_{50} 217 ± 167 μM) and 200% (EC_{50} 8.1 ± 0.1 μM), respectively. Methohexitone, over a wide range of concentration (10–300 μM), had no effect on glycine-evoked currents (Fig. 2). No glycine-mimetic effect was observed with any of these anaesthetics at the concentrations used.

The potentiation of the response to glycine by thiopentone appeared to plateau, at approximately 250%, at a thiopentone concentration between 20 and 30 μM. This is in contrast to previous findings where, although the potentiation by 10 μM thiopentone was of the order of 200%, higher concentrations of thiopentone produced greater potentiation, up to 1400% at 200 μM (Daniels and Roberts, 1998). Pentobarbitone, at 400 μM, potentiated the response to glycine by some 400%, with no clear evidence that this represented a maximal effect. This is in close agreement with previous work (Daniels and Roberts, 1998) and also indicates that the EC_{50} estimated (217 μM) is probably low, consistent with that reported previously, 845 μM (Belelli et al., 1999). The failure of methohexitone to affect the response to glycine mirrored the lack of effect of phenobarbitone reported previously (Daniels and Roberts, 1998).

Propofol potentiated the current response to 50 μM glycine by 10% at 1 μM and by approximately 600% at 100 μM propofol, with an estimated EC_{50} of 48 μM. Within the clinical range for propofol (2–10 μM), the potentiation averaged 115% (Fig. 2).

Propofol potentiated the response to glycine by some 600%, at 100 μM, which again is less than that reported previously, 2000% (Daniels and Roberts, 1998). However, it is clear that the potentiation observed at 100 μM was not maximal. The estimated EC_{50} (48 μM) is somewhat higher than that reported previously, 16 μM (Belelli et al., 1999). It is possible that this apparent difference in potency arises from a variation in the extent of de-folliculation of the oocytes, which, in this study, was not quantified. Propofol is extremely lipid soluble and is given in an Intralipid suspension. The actual free-aqueous concentration is likely to be very sensitive to partitioning between the Intralipid and lipids in the follicular cells and the oocyte itself. This partitioning is also likely to be sensitive to temperature. Since the concentration–response curve for propofol is very steep (Fig. 2), relatively small variations in free-aqueous concentration might have a large effect on the response observed. It is possible that variation in free-aqueous concentration also underlies the difference in potentiation seen

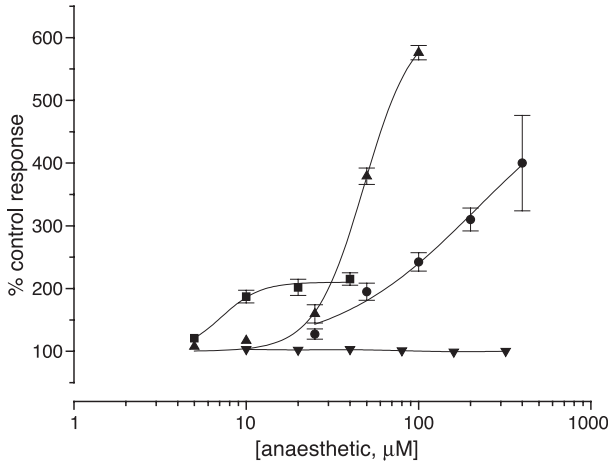

Fig. 2. The percentage of the control current response (recorded in response to the application of 50 μM glycine alone) recorded from oocytes, expressing human α_1 glycine receptors, voltage clamped at −60 mV in response to bath application of 50 μM glycine in standard frog Ringer in the presence of anaesthetics: thiopentone (■), pentobarbitone (●), methohexitone (▼) and propofol (▲). In all cases, the data points represent the mean for six experiments and the bars the standard error of the mean. The lines for thiopentone, pentobarbitone and propofol represent the best fit to a logistic function (see text). Thiopentone: EC_{50} 8.1 ± 0.1 μM, h 3.5 ± 0.1, min 100 (fixed) and max 254 ± 1.2. Pentobarbitone: EC_{50} 217 ± 167 μM, h 1.0 ± 0.3, min 100 (fixed) and max 555 ± 166. Propofol EC_{50} 48 ± 2 μM, h 3.0 ± 0.3, min 100 (fixed) and max 627 ± 24.

with thiopentone, given the lipid solubility of thiopentone, in this study, compared to the previous study.

3.2. Binary combination of thiopentone, pentobarbitone and propofol with methohexitone

The effects of binary combinations of methohexitone with thiopentone, pentobarbitone and propofol on the response to glycine (50 μM) are shown in Fig. 3. Methohexitone (10 μM) decreased the potentiation by thiopentone, in a statistically significant fashion ($p < 0.05$) by 27%, 18% and 13% for the three higher concentrations of thiopentone, 10, 20 and 40 μM, with respect to the equivalent response in the absence of methohexitone. The potentiation of the glycine response by pentobarbitone was also decreased in the presence of methohexitone (10 μM), in a statistically significant fashion ($p < 0.05$) by 18%, 29% and 22%, for 25, 50 and 100 μM pentobarbitone, respectively, with respect to the equivalent response in the absence of methohexitone.

Methohexitone (250 μM) also decreased the potentiation by propofol, in a statistically significant fashion ($p < 0.05$) by 44% and 20% for 50 and 100 μM propofol, with respect to the equivalent response in the absence of methohexitone.

3.3. Binary combinations of thiopentone, pentobarbitone and propofol

The interaction between thiopentone (5–40 μM) and pentobarbitone (50 μM) is illustrated in Fig. 4. Pentobarbitone increased the potentiation by thiopentone at all concentrations, significantly ($p < 0.05$) for the three highest concentrations of thiopentone, by 25%, 19% and 21% (with respect to the potentiation by thiopentone alone) for 10, 20 and 40 μM thiopentone, respectively. The interaction between thiopentone and pentobarbitone appears to be somewhat less than additive, since the responses at 5, 10 and 20 μM thiopentone might have been expected to be increased by at least the 50% potentiation elicited by the 50 μM pentobarbitone alone.

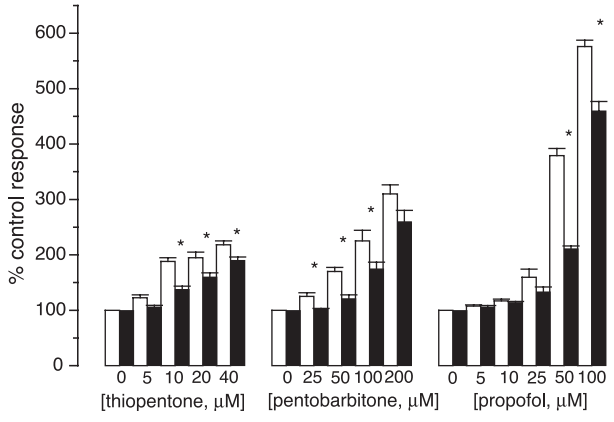

Fig. 3. The effect of methohexitone on the percentage of the control current response (recorded in response to the application of 50 μM glycine alone) recorded from oocytes, expressing human α_1 glycine receptors, voltage clamped at −60 mV to bath application of 50 μM glycine in standard frog Ringer in the presence of anaesthetics thiopentone, pentobarbitone and propofol. In all three cases, the open bars represent the potentiation by the single anaesthetic and the solid bars represent potentiation by the anaesthetics in the presence of methohexitone; 10 μM methohexitone in the case of thiopentone and pentobarbitone and 250 μM methohexitone in the case of propofol. The bars represent the mean from four independent experiments and the error bars show the standard error of the mean. *Denotes statistically significant differences ($p < 0.05$; ANOVA and Student's *t*-test) between the potentiation by the anaesthetic compared to that by the anaesthetic in the presence of methohexitone.

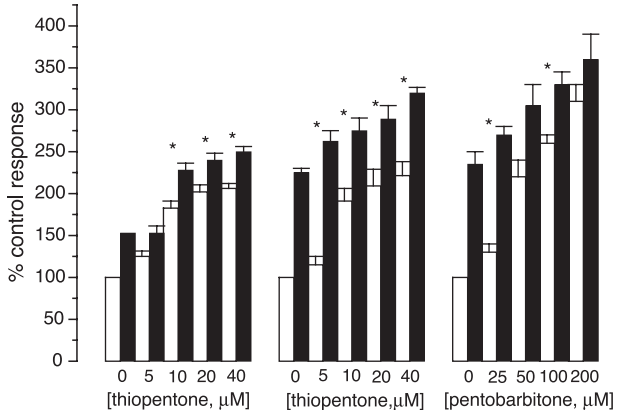

Fig. 4. The effect of pentobarbitone (50 μM) and propofol (25 μM) on the percentage of the control current response (recorded in response to the application of 50 μM glycine alone) recorded from oocytes, expressing human α_1 glycine receptors, voltage clamped at −60 mV to bath application of 50 μM glycine in standard frog Ringer in the presence of anaesthetics thiopentone and thiopentone and pentobarbitone, respectively. In all three cases, the open bars represent the potentiation by the single anaesthetic and the solid bars represent potentiation by the anaesthetics in the presence of pentobarbitone (first panel) or propofol (second and third panels). The bars represent the mean from four independent experiments and the error bars show the standard error of the mean. *Denotes statistically significant differences ($p < 0.05$; ANOVA and Student's *t*-test) between the potentiation by the anaesthetic compared to that by the anaesthetic in the presence of pentobarbitone (first panel) or propofol (second and third panels).

The effect of 25 μM propofol on the potentiation of glycine (50 μM) by thiopentone (5–40 μM) is shown in Fig. 4. Propofol increased the potentiation by thiopentone at all concentrations used ($p < 0.05$), 128%, 44%, 38% and 44% (with respect to the potentiation by thiopentone alone) at 5, 10, 20 and 40 μM thipentone, respectively. Again the combined interaction appears less than additive considering the 130% potentiation by 25 μM propofol alone.

The effect of 25 μM propofol on the potentiation of glycine (50 μM) by pentobarbitone (50–200 μM) is shown in Fig. 4. Propofol significantly ($p < 0.05$) increased the potentiation by pentobarbitone at the three lower concentrations, 108%, 39% and 27% (with respect to the potentiation by pentobarbitone alone) at 25, 50 and 100 μM pentobarbitone, respectively, again in a somewhat less than additive manner given the potentiation by 25 μM propofol alone (130%).

4. Discussion

In this study, we investigated the effects of thiopentone, pentobarbitone, methohexitone and propofol individually and in binary combinations on the homomeric α_1 glycine receptor expressed in *Xenopus* oocytes to test whether binary combinations of anaesthetics act in an additive manner.

The additive action of anaesthetics is well-recognized in clinical anaesthesia (Vinik et al., 1999; Jones et al., 1999) and used so that individual anaesthetic dosages can be reduced, while achieving the same anaesthetic effect, thereby reducing unpleasant anaesthetic-related side-effects and perhaps shortening recovery times (Vuyk et al., 1995). However, it is not known whether anaesthetics act additively at a common receptor site or if the additivity is a physiological summation.

Thiopentone, pentobarbitone and propofol were all found to act as positive allosteric modulators for the α_1 glycine receptor, when activated by low (with respect to the EC_{50}) concentrations of glycine, in confirmation of the previous reports (Daniels and Roberts, 1998; Belelli et al., 1999). This present study has demonstrated that methohexitone, in contrast, is unable to modulate receptor function and, in this respect, behaves as phenobarbitone has previously been reported to act (Daniels and Roberts, 1998). Methohexitone and phenobarbitone may fail to allosterically modulate the glycine receptor because they do not bind at the glycine receptor. Alternatively, they may be incapable, once bound, of affecting the conformational change associated with glycine binding. Since we have shown in this study that methohexitone clearly affects the ability of thiopentone, pentobarbitone and propofol to allosterically modulate the action of glycine at the glycine receptor (Fig. 3), then we must presume that methohexitone is binding at the receptor. The molecular structure of the four barbiturates is similar and the most economical assumption is that they share a common binding site at the glycine receptor. The lack of effect of methohexitone and phenobarbitone at the receptor would, therefore, arise due to a failure to affect the conformational rearrangement induced by glycine.

Methohexitone decreased the potentiation of the response to glycine caused by propofol in a similar manner to its effect on the action of thiopentone and pentobarbitone. By extension of the argument above, we might presume that propofol therefore shares a common binding site to that of the barbiturates. We recognize that an alternative explanation is that methohexitone binds at a different site on the glycine receptor and that in so doing alters the conformation of the receptor such that the affinity of the receptor for thiopentone, pentobarbitone and propofol is reduced.

In support of the concept of a common site of action for the barbiturates and propofol at the α_1 glycine receptor, we have demonstrated that pentobarbitone enhances the potentiation of the action of glycine caused by thiopentone and that propofol enhances the potentiation caused by both thiopentone and pentobarbitone. This is consistent with the concept that these anaesthetics are sharing a common site at which they act to allosterically modulate the action of glycine at its receptor. Clearly, the efficacy by which these anaesthetics act differs and it is this we suggest that makes the combined action appear less than additive in this study.

Our conclusion is also supported by the previous studies, which reported that binary combination of propofol and pentobarbitone, at their maximum effective concentrations (100 μM and 3 mM, respectively), potentiated the response to glycine at the homomeric α_1 receptor to the same extent as 100 μM propofol alone and slightly more than 3 mM pentobarbitone alone (Pistis et al., 1997).

A common site of action for *n*-alcohols and volatile anaesthetics at the α_1 glycine receptor has been reported. Initially, it was reported that potentiation of the action of glycine by *n*-alcohols and volatile anaesthetics requires specific amino acids in the second and third transmembrane segments of the glycine receptor (Mihic et al., 1997). More recently, it was shown that the serine residue S267 in the second transmembrane segment of the α_1 glycine receptor does represent a common binding site for alcohols and volatile anaesthetics (Krasowski and Harrison, 2000). In contrast, the alanine residue in the third transmembrane segment (A288) does not appear to be a common binding site (Mascia et al., 2000). The question arises therefore, whether the common site at which pentobarbitone, thiopentone, methohexitone and propofol are acting is the same as that shared by the *n*-alcohols and volatile anaesthetics. Experiments have suggested that alphaxalone does not share the binding site occupied by alcohols and volatile anaesthetics (Mihic et al., 1997; Rick et al., 1998). We suggest, therefore, that it is possible that a number of different binding sites exist on the glycine receptor, perhaps three, one for alcohols and volatile anaesthetics, one for barbiturates and closely related anaesthetics and a third for steroids and related molecules. Thus, for anaesthetics sharing a binding site, the clinically observed effect of anaesthetic additivity could arise from a common action at one site but for anaesthetics of diverse structure, the additive actions arise through a physiological summation.

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