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Propofol and flurazepam act synergistically to potentiate $GABA_A$ receptor activation in human recombinant receptors

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Abstract

The intravenous general anaesthetic propofol (2,6-di-isopropylphenol) is frequently combined with a benzodiazepine. There are clinical reports of synergism between these two agents for induction of general anaesthesia. To investigate a possible mechanism of this synergistic interaction between propofol and benzodiazepines, the effect of propofol and flurazepam on $GABA_A$ receptor function was examined in *Xenopus* oocytes expressing human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs. Potentiation of GABA receptor-activated current by low (1–10 μM) concentrations of propofol together with flurazepam (0.25–0.5 μM) was significantly greater than predicted by an additive response. Isobolographic analysis indicated a strong synergistic interaction between propofol and flurazepam at either of the receptor constructs examined. In contrast, the cyclopyrrolone derivative zopiclone, which produced a similar facilitation of GABA receptor-activated current compared to flurazepam, produced a less than additive potentiation when combined with propofol. Flurazepam significantly decreased the EC_{50} concentration of propofol for potentiation of GABA responses. Thus, flurazepam, in addition to facilitating GABA receptor activity on its own, also increases the apparent affinity of the $GABA_A$ receptor complex to propofol, resulting in a greater than expected potentiation by the combination of propofol plus flurazepam.

Keywords: Propofol; Benzodiazepine; $GABA_A$ receptor; Flurazepam; Anesthesia; *Xenopus* oocyte

1. Introduction

Potentiation of inhibitory synaptic transmission mediated by γ-aminobutyric acid (GABA) acting at the $GABA_A$ receptor subtype is increasingly being regarded as a primary mechanism of action for drugs with hypnotic, anaesthetic, and anticonvulsant properties. Propofol (2,6-di-isopropylphenol) is an intravenous general anaesthetic which is chemically unrelated to other agents, and is favoured because of a rapid recovery from anaesthesia with minimal residual effects (O'Toole et al., 1987; MacKenzie and Grant, 1985). A significant body of evidence has accumulated to suggest that propofol acts as a positive modulator of the $GABA_A$ receptor. Thus, propofol at pharmacologically relevant concentrations increases the binding of [^{3}H]GABA, reduces the binding of *t*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS), enhances GABA- and muscimol-stimulated $^{36}Cl^-$ uptake, and potentiates membrane current activated by exogenous and synaptically released GABA (Concas et al., 1991; Orser et al., 1994; Peduto et al., 1991).

Propofol is often used in combination with benzodiazepines, either to enhance anaesthesia or to reduce preoperative anxiety (Bailie et al., 1989; Short and Chui, 1991). Propofol and midazolam were found to act synergistically for the induction of anaesthesia (Short and Chui, 1991). Similarly, temazepam significantly increased recovery time in patients anaesthetized with propofol and alfentanil (Bailie et al., 1989). An interaction at the $GABA_A$ receptor-Cl^- channel complex was suggested, but alterations in drug disposition could not be excluded. The purpose of this study therefore was to investigate the effects of propofol on $GABA_A$ receptor function in the presence and absence of a benzodiazepine receptor agonist. Two different receptor constructs ($\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$) of the $GABA_A$ receptor were expressed in *Xenopus* oocytes. In addition, the effects of propofol on GABA receptor-activated current was also examined in $\alpha_2\beta_2$ receptors, to assess the influence of the γ_2 subunit in propofol potentiation of $GABA_A$ receptor function. The results of this study suggest that propofol and benzodiazepines interact at the receptor level to facilitate $GABA_A$ receptor-activated Cl^-

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current. Moreover, this interaction may be specific for classical benzodiazepine receptor agonists, since the non-benzodiazepine cyclopyrrolone derivative zopiclone, considered a full benzodiazepine receptor agonist, produced a less than additive response when combined with propofol.

2. Materials and methods

2.1. Preparation of oocytes

Female *Xenopus laevis* frogs (Xenopus) were anaesthetized by immersion in ice-cold water containing 0.2% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co.), and 1–2 pieces of ovary were removed through a small incision in the abdomen. Stage V and VI oocytes were isolated, rinsed and stored in a buffered saline solution containing (in mM): 88 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2.4 $NaHCO_3$, 5 Hepes, 2 sodium pyruvate, 0.5 theophylline, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), pH 7.4. The theca, epithelial and follicular layers were dissected away manually using fine forceps.

DNAs (cDNAs) for the human α_1, α_2, β_2 and γ_{2L} subunits of the $GABA_A$ cloned into the pCDM8 vector (Invitrogen) were linearized with appropriate restriction enzymes, and cRNAs transcribed and end-capped in vitro using the RiboMAX Large Scale RNA production system (Promega). Individual oocytes were injected with 20–40 nl of a cRNA mixture composed of $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, or $\alpha_2\beta_2$ (0.8–1 ng/nl of each subunit) using a Drummond Nanoject Automatic Injector (Drummond Scientific) and incubated at room temperature (20–22°C) for 2–3 days prior to electrophysiological recording.

2.2. Electrophysiological recording and data analysis

Single oocytes were placed, animal pole up, in a square of nylon mesh (1.5 mm × 1.5 mm) glued to the glass bottom of a flow chamber (RC recording chamber, Warner Instruments, 8 mm well size). Oocytes were impaled with two microelectrodes containing 3 M KCl (2–4 MΩ) and voltage-clamped at −70 mV using a Warner Instruments OC-725B Oocyte Clamp and perfused at 6–8 ml/min with a buffered saline solution containing (in mM): 88 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.4. Currents in response to application of GABA or GABA plus drugs were amplified and recorded on a strip chart recorder for later analysis. Choice of solutions was controlled by a series of manual valves connected to the inflow of the recording chamber. GABA, or GABA plus drugs, was

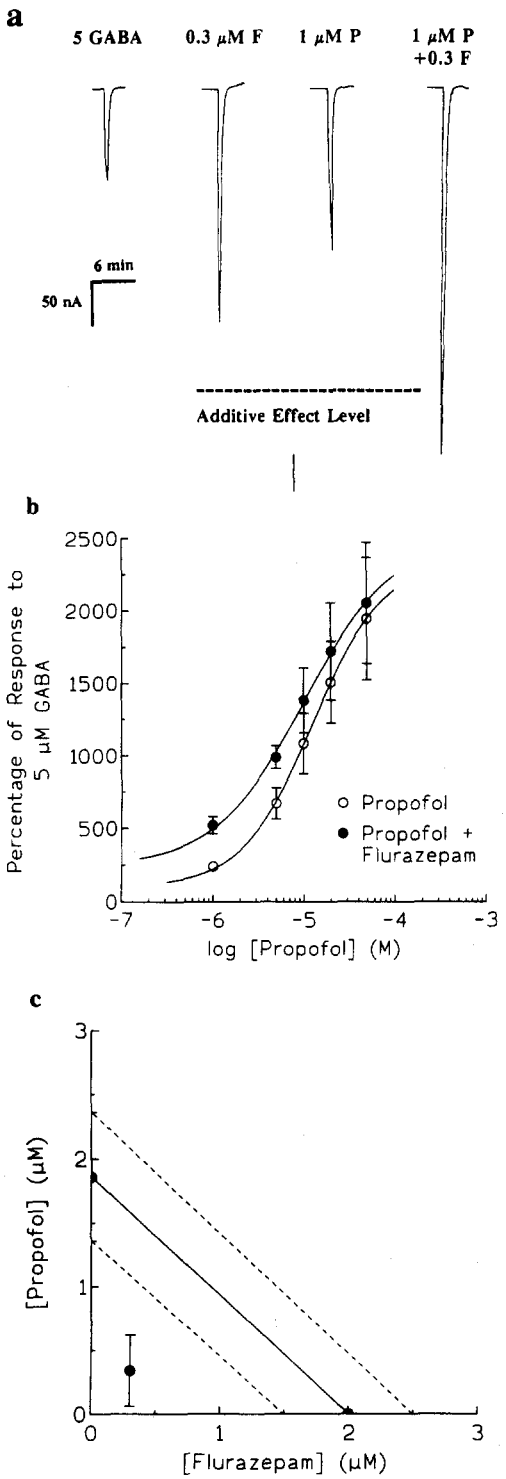

Fig. 1. Propofol and flurazepam act synergistically on $GABA_A$ receptors. (a) Current responses in an oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ subunits of the $GABA_A$ receptor. The response to 5 μM GABA was potentiated by both 0.3 μM flurazepam and by 1 μM propofol. The combination produced a large potentiation of the GABA response. The theoretical additive current response is shown for comparison. (b) Cumulative data ($n = 5$) for the interaction of 0.3 μM flurazepam with propofol (1–50 μM) on $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Flurazepam shifted the concentration-response relationship for propofol to the left, without changing the maximal response. (c) Isobologram for the interaction of propofol and flurazepam. The theoretical additive isobole for a 300% increase in the control response is shown as a solid line. Dashed lines indicate the 95% confidence limits. The combination of propofol and flurazepam which produces this effect (with 95% confidence limits) is also shown. The interaction term is approximately 0.35, which indicates synergism betweem these two drugs.

applied to the oocyte until the steady-state peak response was obtained (30–60 s), with an interval of 5–15 min between applications. To examine the interaction between propofol and flurazepam to modulate $GABA_A$ receptor function, a theoretical additive isobole for flurazepam and propofol was constructed from the concentration-response relationships of each drug alone. An effect level of 300% increase in the control reponse to 5 μM GABA was chosen arbitrarily, as this point falls within the linear region of the concentration-response relationship for each drug alone. In other experiments, a theoretical additive response was calculated from the individual current responses as the sum of the current increase over control (see Fig. 1). This was then compared to the actual degree of potentiation of the GABA response obtained when the two drugs were combined (the experimental response). Concentration-response relationships for GABA-activated membrane current and potentiation by propofol and flurazepam were fit to the logistic equation $I = I_{max}[C^n/(C^n + EC_{50}^n)]$, to obtain estimates of the maximal response (I_{max}), Hill coefficient (n), and EC_{50} values. The best fit to the data was determined using a nonlinear least-square fitting program (Inplot4, Graphpad Software). Unless otherwise noted, data are expressed as the means ± S.E.M. Where appropriate, either repeated measures analysis of variance, or ordinary one-way analysis of variance, coupled with Student Neuman-Keuls post-test for multiple comparisons were used to assess the statistical significance of the data (Instat2, Graphpad Software).

3. Results

Injection of *Xenopus* oocytes with mRNA coding for subunits of the human $GABA_A$ receptor resulted in the expression of robust GABA-activated membrane currents. Receptors composed of $\alpha_1\beta_2\gamma_{2L}$ subunits had an EC_{50} for GABA of 58 ± 9 μM and a Hill slope of 1.30 ± 0.07 ($n = 5$). Flurazepam (0.05–10 μM) produced a concentration-dependent increase in the response to 5 μM GABA with an EC_{50} of 0.52 ± 0.11 μM, and a maximal potentiation of 343 ± 12% of the control response ($n = 6$). In contrast, the maximal potentiation of the response to 5 μM GABA with propofol (1–50 μM) was greater than 2300% (Fig. 1b). When combined with 0.3 μM flurazepam, potentiation of the GABA response induced by propofol was greater than expected from a simple additive response (Fig. 1a). The concentration-response relationship for propofol was shifted to the left by flurazepam (Fig. 1b), with no significant change in the values for the maximal response or the Hill slope derived from the logistic equation. The EC_{50} for propofol was decreased from 10 ± 1 μM to 5 ± 1 μM by 0.3 μM flurazepam ($n = 5$, $P < 0.01$). Using an effect level of 300% of the control response, a theoretical additive isobole was constructed for propofol and flurazepam (Fig. 1c). The concentrations of each drug alone producing this effect were 2 ± 0.2 μM ($n = 6$) and 1.9 ± 0.2 μM ($n = 5$) for flurazepam and propofol, respectively. The concentration of propofol required to achieve this effect calculated from the concentration-response curve for each oocyte in the presence of 0.3 μM flurazepam was 0.34 ± 0.1 μM ($n = 5$), indicating synergy between these two drugs (Fig. 1c).

Direct activation of the $GABA_A$ receptor requires higher concentrations of propofol than those which potentiate the response to GABA (Orser et al., 1994), and this direct effect of propofol can be potentiated by benzodiazepines (Hara et al., 1993). In oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ receptors, the threshold concentration of propofol for direct activation of membrane current was 10 μM (6 ± 3 nA, $n = 5$). Flurazepam at a concentration of 0.5 μM slightly

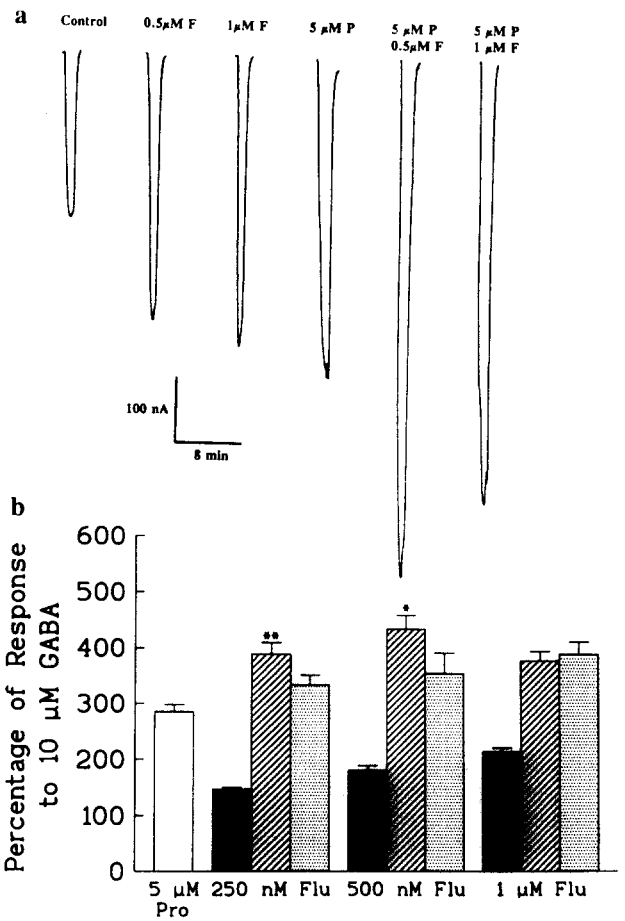

Fig. 2. Effect of flurazepam concentration on the interaction between flurazepam and propofol. (a) Current responses to 10 μM GABA in an oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ subunits of the $GABA_A$ receptor. Flurazepam produces a concentration-dependent enhancement of the GABA response. When co-applied with 5 μM propofol, a greater than additive effect was obtained with 500 nM flurazepam, but not 1 μM flurazepam. (b) Cumulative data showing the degree of potentiation of the response to 10 μM GABA with 5 μM propofol alone (open bar), flurazepam alone (solid bars), propofol plus flurazepam (hatched bars), and the theoretical additive response (speckled bars). Enhancement of the GABA response by flurazepam plus propofol was significantly greater than the theoretical additive response for 250 nM (** $P < 0.01$, $n = 4$) and 500 nM (* $P < 0.05$, $n = 4$) flurazepam, but not 1 μM flurazepam ($n = 5$).

increased this direct propofol-activated current (11 ± 4 nA, $P = 0.051$, paired t-test). However, the interaction between propofol and flurazepam was strongest at concentrations of propofol less than 10 μM (e.g., Fig. 1b), thus it seems unlikely that direct activation of $GABA_A$ receptors by propofol accounts for the observed synergism.

Increasing concentrations of flurazepam (0.25–1 μM) produced increasing degrees of potentiation of the response to 10 μM GABA (Fig. 2a,b). When combined with 5 μM propofol, a greater than additive potentiation of the GABA response was obtained with 0.25 and 0.5 μM flurazepam (Fig. 2b). At 1 μM flurazepam however, the combined response with 5 μM propofol was not different than the predicted additive response (Fig. 2a,b). Co-application of the benzodiazepine receptor partial inverse agonist ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (RO15-4513) at a concentration (0.5 μM) which had little effect on the control GABA response and which completely blocked potentiation by flurazepam, had no effect on the response to propofol (Fig. 3).

There was no difference between $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ receptor constructs for the interaction between flurazepam and propofol to potentiate GABA receptor-activated current (data not shown). $GABA_A$ receptors composed of

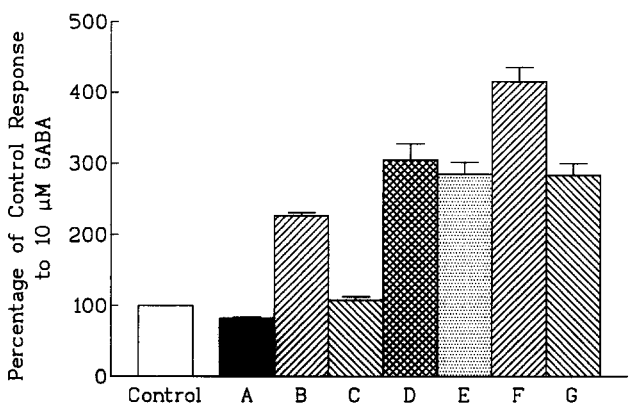

Fig. 3. Potentiation of the GABA response by flurazepam and propofol are differentially sensitive to block by RO15-4513. Cumulative data ($n = 4$) showing potentiation of the response to 10 μM GABA in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor construct by flurazepam and propofol in the presence and absence of 0.5 μM RO15-4513. At this concentration RO15-4513 alone (A) produced a small reduction in the response to 10 μM GABA. Flurazepam at a concentration of 1 μM produced a significant potentiation of the GABA response (B, $P < 0.001$), and this was completely blocked by co-application of 0.5 μM RO15-4513 (C). There was no difference between the amplitude of the GABA response in the presence of RO15-4513 alone (A) and that in the presence of RO15-4513 plus flurazepam (C). Propofol at a concentration of 5 μM produced a robust potentiation of the GABA response (D), which was unaffected by co-application of 0.5 μM RO15-4513 (E). Propofol and flurazepam together produced a potentiation of the GABA response that was greater than either agent alone (F, $P < 0.001$). When 0.5 μM RO15-4513 was co-applied with propofol and flurazepam (G), the resulting potentiation of the GABA response was not different from that obtained with propofol alone or propofol together with RO15-4513 (D and E). Therefore, blocking the benzodiazepine site does not alter the ability of propofol to potentiate the GABA response.

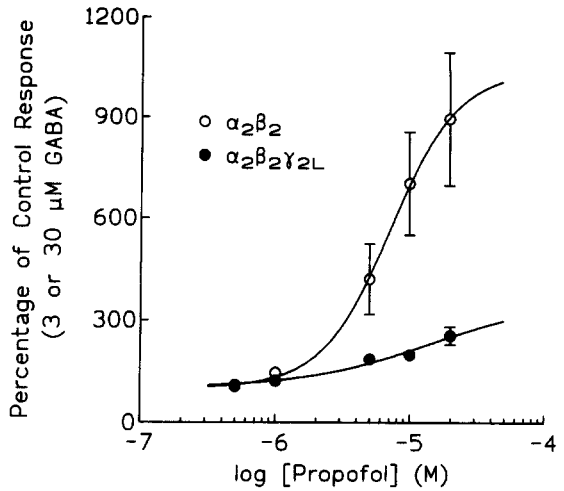

Fig. 4. The γ_{2L} subunit influences propofol potentiation of $GABA_A$ receptor-activated membrane current. Concentration-response relationships for propofol to potentiate GABA receptor-activated membrane current in $\alpha_2\beta_2$ (open circles) and $\alpha_2\beta_2\gamma_{2L}$ (closed circles) receptor constructs are shown. Equivalent concentrations (the approximate EC_{30} values) of 3 and 30 μM GABA were used on $\alpha_2\beta_2$ and $\alpha_2\beta_2\gamma_{2L}$ receptors, respectively. Propofol produced a greater maximal potentiation of the GABA response in $\alpha_2\beta_2$ compared to $\alpha_2\beta_2\gamma_{2L}$ receptors. The EC_{50} for propofol potentiation of the GABA response was significantly less ($P < 0.001$) in $\alpha_2\beta_2$ receptors (7 ± 0.9 μM, $n = 6$) compared to $\alpha_2\beta_2\gamma_{2L}$ receptors (14 ± 0.2 μM, $n = 6$).

$\alpha_2\beta_2\gamma_{2L}$ subunits responded to GABA with an EC_{50} of 67 ± 4 μM and a Hill slope of 1.12 ± 0.05 ($n = 5$), and had similar sensitivities to propofol and flurazepam. A concentration of 0.5 μM flurazepam shifted the EC_{50} for propofol potentiation of the 3 μM GABA-activated membrane current from 16 ± 4 μM to 4 ± 0.7 μM ($n = 7$, $P < 0.01$).

The γ_2 subunit is required for benzodiazepines to potentiate GABA receptor function. That is, in the absence of a γ subunit, benzodiazepines do not enhance GABA receptor-activated membrane current (Knoflach et al., 1992; Sigel et al., 1990). To assess the role of the γ_{2L} subunit for propofol potentiation of $GABA_A$ receptor function, we compared $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ receptor isoforms for sensitivity to propofol. The $\alpha_2\beta_2$ receptor construct had a much greater sensitivity to GABA (EC_{50} was 8 ± 2 μM), as expected for $GABA_A$ receptors without a γ_2 subunit (Horne et al., 1993). We chose to use equivalent concentrations of GABA (3 and 30 μM GABA, the approximate EC_{30} concentrations for $\alpha_2\beta_2$ and $\alpha_2\beta_2\gamma_{2L}$ receptors, respectively) to compare these two receptor constructs. The absence of the γ_{2L} subunit significantly influenced both the efficacy and potency for propofol potentiation of GABA receptor-activated current (Fig. 4). The maximal potentiation of the GABA response produced by propofol (calculated from the logistic equation) was much greater in the $\alpha_2\beta_2$ receptor construct. In addition, the EC_{50} concentration of propofol for potentiation of the GABA response was significantly lower in the $\alpha_2\beta_2$ receptor construct compared to the $\alpha_2\beta_2\gamma_{2L}$ receptor (Fig. 4). Thus, the γ_2

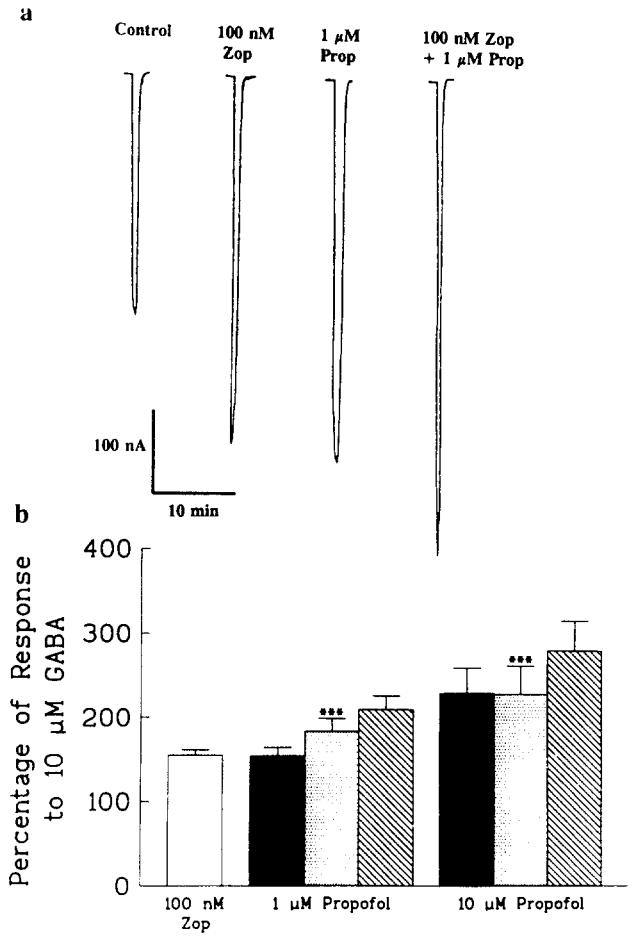

Fig. 5. Zopiclone and propofol produce a less than additive facilitation of $GABA_A$ receptor function. (a) Response to 10 μM GABA in the presence of zopiclone and propofol separately and in combination, in an oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. (b) Cumulative data ($n = 5$–6) for the facilitation of the response to 10 μM GABA by zopiclone alone (open bars), propofol alone (solid bars), propofol plus zopiclone (speckled bars), and the theoretical additive response (hatched bars). Enhancement of the GABA response by propofol plus zopiclone was significantly less than expected from the additive response of each agent alone, *** $P < 0.001$.

subunit, while not an absolute requirement, does appear to play a role in determining the potency and efficacy for propofol potentiation of $GABA_A$ receptor function.

Zopiclone is a cyclopyrrolone derivative which is reported to potentiate $GABA_A$ receptor activity similarly to the benzodiazepines. The interaction between zopiclone and propofol was therefore tested on the $\alpha_1\beta_2\gamma_{2L}$ subunit combination. Zopiclone (0.05–5 μM) increased the response to 10 μM GABA with an EC_{50} for zopiclone of 107 ± 9 nM ($n = 9$). The maximal potentiation of the GABA response obtained with zopiclone was the same as that obtained with 10 μM flurazepam, a maximally effective concentration of this benzodiazepine (data not shown). When zopiclone (100 nM) was combined with propofol (1–10 μM), the resulting potentiation of the response to 10 μM GABA was significantly lower than the expected additive response (Fig. 5).

4. Discussion

In this study, propofol was found to robustly potentiate the response to GABA on $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_2\beta_2$ subtypes of the $GABA_A$ receptor. The concentrations used (0.5–50 μM) span the range of whole blood propofol concentrations during the induction and maintenance phases of anaesthesia (Schuttler et al., 1988). When propofol was combined with midazolam, a potent, water-soluble benzodiazepine, a synergistic interaction was found for both hypnosis and anaesthesia (Short and Chui, 1991). For hypnosis there was a 44% reduction in the ED_{50} concentration for each agent acting individually. For anaesthesia, midazolam reduced the ED_{50} for propofol by over 50%. Moreover, although midazolam was unable to induce anaesthesia on its own it behaved as an equi-effective anaesthetic agent when combined with propofol (Short and Chui, 1991). In our study, when combined with flurazepam (a full agonist at the benzodiazepine receptor), the potentiation of $GABA_A$ receptor activity obtained with propofol was greater than expected from a simple additive response. The EC_{50} concentration of propofol for potentiation of the GABA response was significantly reduced by flurazepam. Isobolographic analysis suggests a strong synergistic interaction between propofol and flurazepam. Thus, the reported clinical synergism between propofol and benzodiazepines can be replicated at the level of the $GABA_A$ receptor in vitro, which underscores the significance of this receptor mechanism for anaesthesia.

Flurazepam produced a significant decrease in the EC_{50} concentration for propofol without increasing the maximal potentiation of the GABA response obtained with propofol. Thus, it appears that flurazepam (and presumably other benzodiazepines), in addition to facilitating $GABA_A$ receptor activity on its own, also increases the apparent affinity of the receptor for propofol, resulting in a greater than expected potentiation of the GABA response by lower concentrations of propofol. Given the importance of the γ_2 subunit for benzodiazepine modulation of $GABA_A$ receptor function (Knoflach et al., 1992; Sigel et al., 1990), and the influence of this same subunit in determining the efficacy and potency of propofol to potentiate $GABA_A$ receptor function (Fig. 4), it is tempting to speculate that the γ_2 subunit plays a role in the interaction between propofol and benzodiazepines. However, further experiments will be needed to address this question.

Hara et al. (1993) demonstrated that diazepam, but not pentobarbital, could facilitate a propofol-induced current in acutely dissociated hippocampal neurons, which supports the idea of a selective interaction between propofol and benzodiazepines. Propofol appears to bind to a site on the $GABA_A$ receptor which is distinct from the binding site for barbiturates, neurosteroids and alphaxolone (Concas et al., 1991; Prince and Simmonds, 1992a), and which is insensitive to benzodiazepine receptor antagonists (Concas et al., 1991; Hara et al., 1993; Peduto et al., 1991).

Similarly, we have found that RO15-4513, a partial inverse agonist at the benzodiazepine site, completely abolishes the modulatory effect of flurazepam on $GABA_A$ receptor function but has no influence on the facilitatory effect of propofol.

Zopiclone, classified as a full agonist at the benzodiazepine site (Doble et al., 1992), is a potent allosteric modulator of $GABA_A$ receptor function and is known to have hypnotic and anxiolytic properties similar to benzodiazepines. Zopiclone produced a concentration-dependent facilitation of GABA responses in oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor construct, with an efficacy equal to a maximally effective concentration of flurazepam. Cyclopyrrolones such as zopiclone inhibit the binding of [^{3}H]benzodiazepine receptor agonists and antagonists to brain membranes (Blanchard et al., 1979; Concas et al., 1994). However, there is evidence that cyclopyrrolones and benzodiazepines bind to distinct, but allosterically coupled, sites on the $GABA_A$ receptor, and may also induce different conformational states of the receptor (Blanchard et al., 1983; Doble, 1983; Prince and Simmonds, 1992b; Trifiletti and Snyder, 1984). In our study, the combination of zopiclone and propofol produced an enhancement of the response to 10 μM GABA which was significantly less than the theoretical additive response. This might be due to some overlap in the binding sites for propofol and zopiclone on $GABA_A$ receptors. Alternatively, the conformational change induced in the receptor by zopiclone may inhibit the effects of propofol, producing a negative allosteric interaction between these two drugs. From the results with zopiclone it would appear that facilitation of the GABA response alone is not sufficient to explain the greater than additive response obtained with propofol and flurazepam. That is, the interaction between propofol and benzodiazepines may be specific to these classes of drugs, and not generalized to all substances which act at or near the benzodiazepine site to facilitate $GABA_A$ receptor function.

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