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Triton X-100 inhibits agonist-induced currents and suppresses benzodiazepine modulation of $GABA_A$ receptors in *Xenopus* oocytes

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ABSTRACT

Changes in lipid bilayer elastic properties have been proposed to underlie the modulation of voltage-gated Na^+ and L-type Ca^{2+} channels and $GABA_A$ receptors by amphiphiles. The amphiphile Triton X-100 increases the elasticity of lipid bilayers at micromolar concentrations, assessed from its effects on gramicidin channel A appearance rate and lifetime in artificial lipid bilayers. In the present study, the pharmacological action of Triton-X 100 on $GABA_A$ receptors expressed in *Xenopus laevis* oocytes was examined. Triton-X 100 inhibited $GABA_A$ $\alpha_1\beta_3\gamma_{2S}$ receptor currents in a noncompetitive, time- and voltage-dependent manner and increased the apparent rate and extent of desensitization at 10 μM, which is 30 fold below the critical micelle concentration. In addition, Triton X-100 induced picrotoxin-sensitive $GABA_A$ receptor currents and suppressed allosteric modulation by flunitrazepam at $\alpha_1\beta_3\gamma_{2S}$ receptors. All effects were independent of the presence of a γ_{2S} subunit in the $GABA_A$ receptor complex. The present study suggests that Triton X-100 may stabilize open and desensitized states of the $GABA_A$ receptor through changes in lipid bilayer elasticity.

1. Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. The inhibitory action is mediated via activation of metabotropic $GABA_B$ and ionotropic $GABA_A$ receptors, respectively. The fast inhibitory GABAergic synaptic transmission is primarily controlled by the chloride conducting $GABA_A$ receptors [1]. These receptors belong to the Cys-loop superfamily of ligand-gated ion channels that includes the glycine receptor, the nicotinic acetylcholine (nACh) receptor, the 5-hydroxytryptamine ($5\text{-}HT_3$) receptor, and the recently identified zinc-activated ZAC receptor [2].

Pharmacological modulation of the $GABA_A$ receptors has been studied in great detail. However, it is poorly characterized how the lipid bilayer-embedded receptor is modulated by the physical properties of the cell membrane, lipids, and membrane-perturbing amphiphilic compounds. An increasing number of studies show that the lipid composition of the membrane strongly influences the function of bilayer-embedded proteins [3–7]. The incorporation of cholesterol or free fatty acids can significantly affect the activity of integral membrane proteins. Furthermore, changes in lipid characteristics, such as head group type, fatty acyl chain length or degree of unsaturation strongly modulate the activity of integral membrane receptors. However, the molecular mechanisms underlying such lipid–protein interactions have been difficult to uncover. Accumulating evidence suggests that lipids can modulate protein function via "nonspecific" mechanisms involving changes in the biophysical properties of the membrane bilayer rather than direct lipid–protein interactions [8–12].

The best documented nonspecific lipid–protein interactions are those that result from a hydrophobic coupling between the hydrophobic length of the protein and the hydrocarbon thickness of the surrounding lipid bilayer, which exists to avoid a lipid–protein hydrophobic mismatch [13–17]. One model proposes that regulation of protein function could result from such protein–bilayer hydrophobic interactions that couple protein conformational changes to elastic deformations of the surrounding membrane bilayer [13–15,17,18]. The elastic properties of a membrane bilayer can be modulated by amphiphiles, and changes in elasticity can be assessed using gramicidin A channels as force transducers [19]. Indeed, specific amphiphiles chosen due to their ability to alter membrane elasticity also modulate the function of voltage-gated Na^+ and L-type Ca^{2+} channels and of $GABA_A$ receptors [9,18]. These studies show a close correlation between amphiphile effects on voltage-gated ion channel function and their effects on gramicidin A channel activity, strongly suggesting that changes in bilayer elasticity underlie the channel modulation.

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The nonionic detergent Triton X-100 is one such amphiphilic model compound that increases lipid bilayer elasticity at concentrations 30- to 100-fold below the critical micelle concentration (CMC) [9,18]. Thus, it can be used to investigate the possible influence of bilayer elasticity on a given membrane protein. Interestingly, early studies have shown that Triton X-100 increases specific [^{3}H]-muscimol or [^{3}H]-GABA binding to GABA receptors in membrane preparations obtained from vertebrate brain [20–26]. This enhanced binding has been suggested to be due to the liberation of endogenous inhibitors from the membranes or synaptosomal structures [26–28]. However, we have recently shown that Triton X-100 and other amphiphiles known to affect the elasticity of artificial lipid bilayers modulated agonist binding and electrophysiological properties of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors in a similar manner. The amphiphiles had very different chemical structures and modulated $GABA_A$ receptor function at the concentrations that alter lipid bilayer elasticity [29]. This study strongly suggested that the amphiphiles modulate the function of $GABA_A$ receptors through a bilayer-mediated mechanism analogous to that proposed for voltage-gated ion channels [9,18].

In the present study we performed a more detailed pharmacological investigation of the effects of Triton X-100 on the electrophysiological properties of $GABA_A$ receptor channels at concentrations that increase the elastic properties of lipid bilayers without causing membrane disruption. Recombinant $\alpha_1\beta_3\gamma_{2S}$ or $\alpha_1\beta_3$ $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes and receptor-mediated currents were recorded using the two-electrode voltage-clamp technique. To elucidate the underlying mechanism(s) of action, we also examined the putative allosteric effect of Triton X-100 using the benzodiazepine-site agonist flunitrazepam.

2. Materials and methods

2.1. Chemicals and cDNA

The cDNAs encoding human α_1, β_3 and γ_{2S} $GABA_A$ receptor subunits engineered into a pCDM8 (α_1) or a pcDNAI/Amp (β_3 and γ_{2S}) vector (Invitrogen, San Diego, CA, USA) were gifts from Dr Paul Whiting and are described in [30]. Flunitrazepam was synthesized at H. Lundbeck (Denmark). Triton X-100 (protein-grade, 10% solution) was purchased from Calbiochem (La Jolla, CA, USA) and picrotoxin was from Sigma-Aldrich (Denmark). Drug solutions were prepared by diluting aqueous stock solutions of GABA (0.5 M) and Triton X-100 (10%) and dimethyl sulfoxide (DMSO) stock solutions of flunitrazepam (10 mM) and picrotoxin (50 mM) in the extracellular solution. The final concentration of DMSO did not exceed 0.1% (v/v), which had no effect on currents in $GABA_A$ receptor expressing oocytes.

2.2. Expression of $GABA_A$ receptors in X. laevis oocytes

Surgical removal of oocytes from a *X. laevis* frog and oocyte preparation were performed as previously described [31]. *Xenopus* oocytes were collected under anaesthesia. The protocol complies with the European Community guidelines for the use of experimental animals, and the experiments were approved by The Danish National Committee for Animal Studies. Before cRNA injection, the oocytes were kept for 24 h at 19 °C in Kulori buffer consisting of: 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES (pH 7.4). Capped cRNAs encoding human α_1 and β_3 and γ_{2S} $GABA_A$ receptor subunits were produced by *in vitro* transcription of linearized expression vectors containing α_1, β_3 and γ_{2S} cDNAs using mCAP mRNA Capping kit (Stratagene, La Jolla, CA, USA). Each oocyte was injected with 50 nl of distilled H_2O containing α_1, β_3 and γ_{2S} cRNAs in a ratio of 1:1:1 (approximately 6.67 ng cRNA of each subunit, i.e. 20 ng cRNA in total) using a Nanoject microinjector (Drummond Scientific, USA). For expression of $\alpha_1\beta_3$ receptors, 50 nl cRNA solution pr. oocyte was injected, containing 10 ng cRNA of each subunit. Oocytes were kept at 19 °C in Kulori medium for 3–6 days before the experiments were performed.

2.3. Electrophysiology

Whole-cell GABA-evoked currents were recorded from isolated oocytes 2–7 days after RNA injection using the two-electrode voltage-clamp technique. Intracellular recording pipettes were pulled on a PIP5 vertical micropipette puller (HEKA Elektronics, Germany) and filled with 1 M KCl solution. The microelectrodes had open tip resistances of 0.5–1.5 MΩ. Oocytes were placed in a custom-made recording chamber (20 μl) and continuously superfused (about 3 ml/min) with Na100 buffer, which contained 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 adjusted with NaOH). Drugs were applied in the bath solution using a gravity-driven drug-delivery system (ValveBank8, AutoMate Scientific Inc., CA, USA). Cells were voltage clamped at −60 mV for all experiments unless otherwise stated. The currents elicited in response to the application of drugs were recorded at room temperature (20–23 °C) using a Warner OC 725C amplifier (Warner Instruments Inc, UK) interfaced to a Pentium II-based computer via a Digidata 1322A digitizer (Axon Instruments, Foster City, CA, USA). A washout period of 3–10 min was allowed between each GABA application, depending on the duration and the GABA concentration, to ensure complete recovery from desensitization. When Triton X-100 was applied at a concentration that was 30-fold below the critical micelle concentration (CMC, ~300 μM), no effect was observed on the membrane conductance, and the modulatory effects on $GABA_A$ receptor currents were reversible. Thus, Triton X-100 did not cause membrane damage at this concentration (0.0006% v/v, 10 μM). At a concentration of 100 μM (~3-fold below the CMC), which directly activated picrotoxin-sensitive currents in $GABA_A$-receptor expressing oocytes, Triton X-100 has been shown not to cause any significant changes in passive membrane properties (membrane resistance, membrane capacitance or membrane potential) in noninjected oocytes [32], in agreement with our findings (data not shown).

To verify incorporation of the γ_{2S} into the $GABA_A$ receptor complexes, the sensitivity toward the positive allosteric modulator flunitrazepam and the allosteric inhibitor Zn^{2+} was established. Studies have shown that the absence of a γ_2 subunit renders $\alpha_1\beta_3$ $GABA_A$ receptors insensitive to benzodiazepine-mediated modulation and highly sensitive to Zn^{2+} (IC_{50} 0.1 μM) [33]. Co-expression of α_1, β_3 and γ_{2S} cRNAs resulted in GABA-activated currents that were potentiated by flunitrazepam (1 μM) and had a low sensitivity to inhibition by Zn^{2+} (10 μM), verifying the expression of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors (data not shown).

To determine the action of Triton X-100 on the GABA current–voltage relationship, GABA currents were evoked by 3 μM GABA. At this concentration, receptor desentitization was slow and a steady state current was reached within a few minutes. Upon reaching a steady-state current level, short hyperpolarizing or depolarizing pulses were applied from the holding potential of −60 mV to various test potentials between −100 mV and +60 mV, first in the absence and subsequently in the presence of Triton X-100. The leak (and endogenous) currents at the different membrane potentials were measured in the absence of GABA and subtracted from the GABA-induced currents. All current values were normalized to the GABA-induced steady-state current at −80 mV and plotted as a function of the membrane potential. GABA reversal potentials were determined by linear regression including data points near the *x*-axis intersection of the current–voltage (*I–V*) relationships.

2.4. Data analysis

Data acquisition and analysis were performed with pCLAMP software v8.0 (Axon Instruments, Foster City, CA, USA). Data plotting and statistical analyses were done with Origin v6.1 (OriginLab, MA,

USA) and GraphPad Prism v4.00 (GraphPad Software, CA, USA). Concentration–response curve fitting was done with GraphPad Prism using a nonlinear least square method to fit the data to the Hill equation $I = I_{max}[A]^{n_H}/(EC_{50}^{n_H} + [A]^{n_H})$, where I is the peak amplitude of a current activated by a given concentration of agonist, I_{max} is the maximal current produced by a saturating concentration of agonist ([A]), EC_{50} is the concentration of agonist eliciting a half-maximal response and n_H is the Hill coefficient.

To assess the effect of Triton X-100 on the apparent extent of desensitization for the GABA response in $\alpha_1\beta_3\gamma_{2S}$ or $\alpha_1\beta_3$ receptors, GABA (15 and 5 μM, respectively) was applied for 5 min to allow the desensitization process to reach a steady state. Steady-state currents of these GABA responses were determined by extrapolation using the Levenberg–Marquardt least squares method to fit the desensitization time course to a two- or three component exponential function of the form $I(t) = \Sigma A_i e^{(-t/\tau_i)} + C$, where I is the current at time t; A is the relative amplitude of the component; i is the number of exponential functions; τ is the time constant; and C is the extrapolated steady-state current. Using the fitted steady-state current values, the *observed* extent of desensitization after 5 min of GABA application was estimated to be 94.7 ± 0.7% of the *steady-state* extent for $\alpha_1\beta_3\gamma_{2S}$ receptor responses to GABA alone and 99.4 ± 0.1% for responses to GABA co-applied with Triton X-100 (pre-applied for 60 s) and/or flunitrazepam. Thus, at the end of these GABA applications the current decay was close to steady state, and therefore the ratio of 'end current' to peak current was used to assess the extent of desensitization.

For $\alpha_1\beta_3$ receptor responses to GABA, the corresponding values were 88.8 ± 1.2% and 99.3 ± 0.4% in the absence and presence of Triton X-100, respectively. Thus, the ratio of end current to peak current would slightly underestimate the extent of desensitization for the control responses in these experiments. Notably, for GABA responses in the presence of Triton X-100 the peak current value was likely to be underestimated due to the strongly increased rate of desensitization, which would lead to an underestimation of the extent of desensitization for these responses as well.

Data points are presented in text and figures as mean ± standard error (SE). Statistical differences were determined using a one-way analysis of variance (ANOVA) with Dunnett's and/or Bonferroni's *post hoc* tests or a Student's *t*-test, at a significance level of $p<0.05$.

3. Results

3.1. Functional properties of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors

Application of 15 μM GABA to *Xenopus* oocytes injected with cRNA encoding the $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptor subunits activated fast inward currents that desensitized rapidly (Fig. 1A). Flunitrazepam (1 μM) and diazepam (1 μM) both enhanced the response, whereas $ZnCl_2$ (10 μM) had little effect (data not shown), indicating that the responses indeed were mediated by $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors. The EC_{50} and Hill coefficient for the GABA concentration–response relationship were 10.1 μM (95% CI: 9.0–11.5 μM) and 1.1 ± 0.1, respectively (Fig. 2). This experimental system was used throughout the study unless otherwise stated.

3.2. Triton X-100 suppresses peak currents and increases the apparent rate of desensitization of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors

In subsequent studies, the effects of Triton X-100 (10 μM) on $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors were investigated. Fig. 1 shows that co-application of GABA (15 μM) and Triton X-100 significantly reduced the peak amplitude (by 26 ± 2%; $p<0.0001$; $n=7$) and increased the

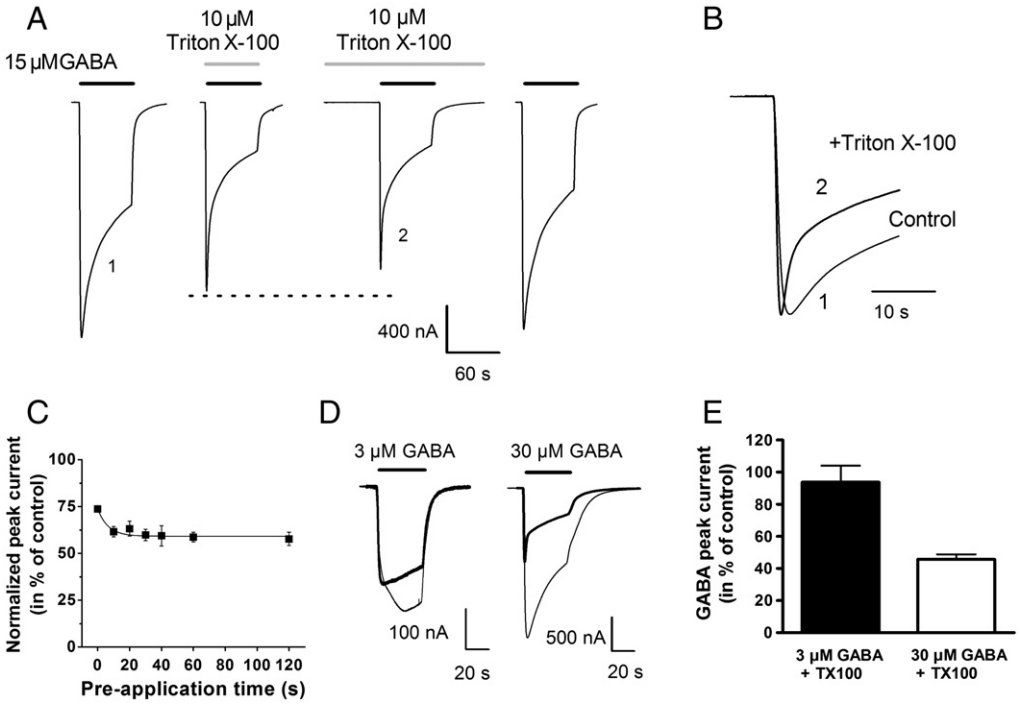

Fig. 1. Time-dependent and reversible effects of Triton X-100 on $GABA_A$ $\alpha_1\beta_3\gamma_{2S}$ receptor currents evoked by GABA. (A) Current traces recorded from a single oocyte showing current responses to 60-s applications of GABA (15 μM) in the absence or presence of 10 μM Triton X-100 at a holding potential of −60 mV. Triton X-100 was either co-applied simultaneously with GABA or pre-applied for 60 s before co-application with GABA. (B) The current evoked by GABA alone and the response to GABA and Triton X-100 (pre-applied) have been normalized with respect to peak current amplitude and superimposed for comparison of the current decay time courses. (C) The peak currents evoked by 15 μM GABA in the presence of Triton X-100 (10 μM) were normalized to the peak currents induced by GABA alone and plotted as a function of the Triton X-100 pre-application time. (D) Current responses to 3 μM GABA (*left panel*) or 30 μM GABA (*right panel*) in the absence or presence of 10 μM Triton X-100. The currents evoked by GABA alone (*thin traces*) are superimposed on the responses to GABA plus Triton X-100 (*thick traces*). Note the different time scales. (E) The effects of Triton X-100 (10 μM) on peak currents induced by 3 μM or 30 μM GABA. Data have been normalized to the control GABA response.

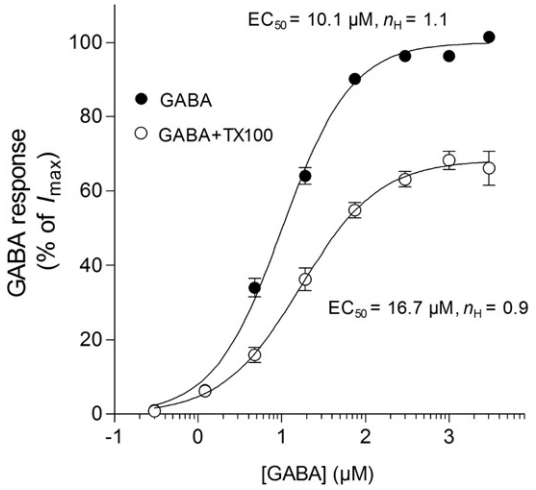

Fig. 2. GABA concentration–response curves of $\alpha_1\beta_3\gamma_{2S}$ receptors in the presence or absence of Triton X-100. GABA was applied in the absence (*black circles*) or presence of 10 μM Triton X-100 (*open circles*) to *Xenopus* oocytes expressing $GABA_A$ receptors with the subunit combination $\alpha_1\beta_3\gamma_{2S}$. Inward currents are normalized to the peak amplitude of currents evoked by a GABA concentration inducing the maximal response (I_{max}). Nonlinear regression analysis of the data sets ($n = 5$–6) was performed as described in the Materials and methods section.

apparent rate of desensitization relative to that of GABA alone. This is in agreement with previously reported data for $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in HEK293 cells [29]. The effects of Triton X-100 were fully reversible (Fig. 1A). A more pronounced effect was observed after pre-application for 60 s of Triton X-100 (Fig. 1, A and C), which led to a 41 ± 3% reduction of the peak amplitude ($p < 0.0001$; $n = 6$), compared to the effects seen with co-application of GABA and Triton X-100 without pre-treatment. As illustrated in Fig. 1C, the relative peak amplitudes after pre-treatment with Triton X-100 for 30, 60 and 120 s did not differ significantly, indicating that the maximal Triton X-100 effect was achieved within the first 30 s of pre-treatment. A 60-s pre-treatment period was used in all subsequent experiments. Since repeated co-application of GABA and Triton X-100 did not result in an increased inhibition of the peak response to GABA, no indication of use dependent block by Triton X-100 was detected (data not shown).

The effect of Triton X-100 on GABA-evoked peak currents was strongly dependent on the GABA concentration (Fig. 1D and E). At a GABA concentration of 3 μM (~3 fold below EC_{50}), Triton X-100 only marginally affected the peak current (6 ± 10% inhibition; $n = 5$), whereas the peak current to 30 μM GABA (~3 fold above the EC_{50}) was reduced by 54 ± 3% ($p < 0.001$; $n = 4$).

3.3. Mechanism of action of Triton X-100 at $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors

To determine whether the inhibitory effects of Triton X-100 on GABA-elicited peak responses were competitive or noncompetitive, GABA concentration–response curves were generated in the absence or presence of 10 μM Triton X-100, respectively (Fig. 2). Triton X-100 resulted in a small, but significant rightward parallel shift ($n_H = 1.1 \pm 0.1$ and 0.9 ± 0.1) of the GABA concentration–response curve (EC_{50} [and 95% CI]: 10.1 [9.0–11.5 μM] to 16.7 [13.0–21.4 μM], $p = 0.0003$) with a suppression of the maximum response to GABA (from 100% to 68 ± 2%, $p < 0.0001$). This suggests a mixed competitive/noncompetitive mechanism of inhibition.

To investigate whether Triton X-100 may interact with $GABA_A$ receptors at a binding site within the channel pore, we determined the effects of the amphiphile on the current–voltage (*I–V*) relationship (Fig. 3). Using a GABA concentration well below the EC_{50} value (3 μM), the *I–V* relationship was determined in the absence and presence of Triton X-100, respectively (10 μM, pre-applied for 1 min). As illustrated in Fig. 1E, Triton X-100 had a marginal effect on the peak amplitude of currents evoked by 3 μM GABA, but a significant suppression was seen with respect to the steady-state current.

The quantitative data showed that Triton X-100 suppressed the GABA-evoked steady-state current at all holding potentials between −100 mV and +60 mV and that the inhibition was significantly more pronounced at positive potentials (Fig. 3A and C). In the presence of Triton X-100, the current level was 38 ± 2% of the control level at −40 mV vs. 20 ± 2% of the control level at +40 mV. As illustrated in Fig. 3A, Triton X-100 had little effect on the reversal potential of the GABA-activated currents (−24 mV and −20 mV in the absence and presence of Triton X-100, respectively). These values correspond

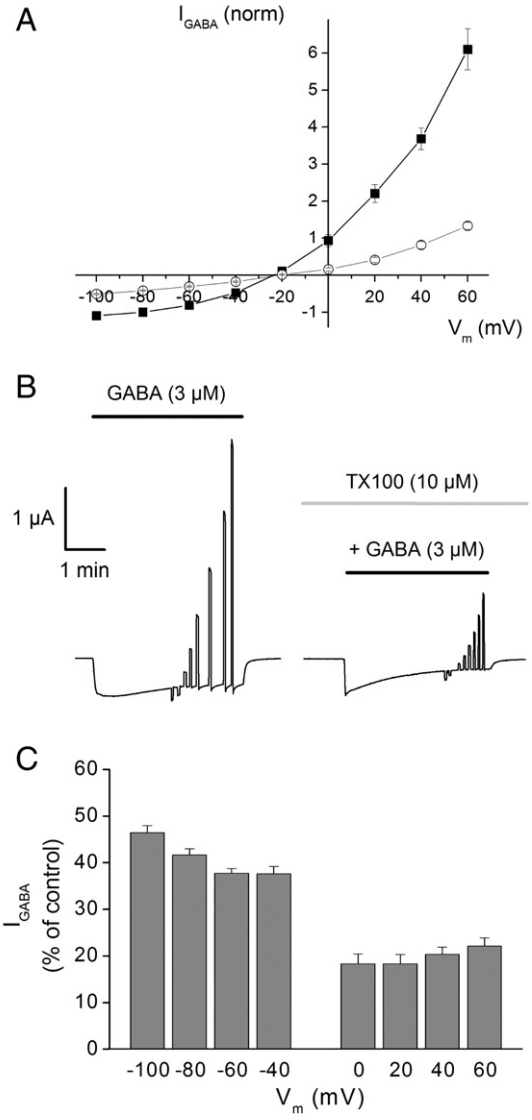

Fig. 3. Triton X-100 suppression of steady-state currents through $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors exhibits voltage dependence. (A) Steady-state values of currents activated by 3 μM GABA are plotted against the holding potential (V_h) in the absence (control, *black circles*) or presence (*open circles*) of 10 μM Triton X-100. All GABA currents have been normalized to the control current value at −80 mV ($n = 3$–6). (B) Current responses to 3 μM GABA in the absence and presence of Triton X-100 (10 μM) in an oocyte voltage-clamped to −60 mV. Upon reaching a steady-state current level, V_h was stepped to potentials between −100 and +60 mV. (C) Quantitative evaluation of the effect of Triton X-100 (10 μM) is presented as percentage of control at holding potentials between −100 and +60 mV. Note that currents at −20 mV have been omitted (−20 mV is close to the reversal potential of $GABA_A$ receptor currents; thus, GABA currents were negligible).

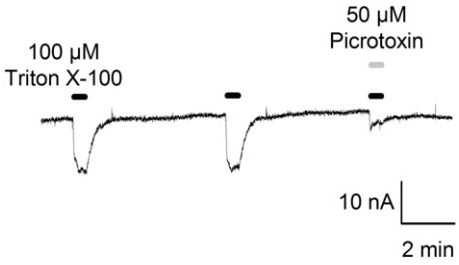

Fig. 4. Activation of picrotoxin-sensitive inward currents by 100 μM Triton X-100. A voltage-clamp recording from a single oocyte expressing $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors is shown ($V_h = -60$ mV). Inward currents were evoked by 100 μM Triton X-100 in the absence and then presence of the $GABA_A$ receptor blocker picrotoxin (PTX, 50 μM).

well with the chloride equilibrium potential in *Xenopus* oocytes in the experimental solution used [34,35], indicating that the detergent did not affect the ionic selectivity of the channels or activate novel non-Cl^- conducting channels.

3.4. Direct effect of Triton X-100 on $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors

On oocytes expressing $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors, low concentrations (≤ 10 μM) of Triton X-100 had only minor or no direct effects on the membrane conductance. However, a slowly activating inward current was observed at a higher concentration (100 μM) of Triton X-100 (Fig. 4), amounting to $0.9 \pm 0.2\%$ of the peak current induced by 1 mM GABA. This current was not observed in noninjected oocytes and could be almost completely inhibited by the $GABA_A$ receptor antagonist picrotoxin (50 μM), suggesting that Triton X-100 at higher micromolar concentrations may mediate activation of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors in the absence of GABA (or change the $GABA_A$ receptor conformation into a conductive state).

3.5. Effect of Triton X-100 on allosteric modulation of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors

To investigate the effects of Triton X-100 on the allosteric properties of the $GABA_A$ receptor, the interaction between Triton X-100 and the benzodiazepine-site agonist flunitrazepam was examined. In agreement with previously described data [36], flunitrazepam (1 μM) enhanced the peak amplitude of currents induced by 15 μM GABA ($162 \pm 11\%$ of control, $p = 0.002$, $n = 6$) (Fig. 5C) and concomitantly increased the rate of desensitization. The potentiation of GABA currents by flunitrazepam was abolished by co-administration of Triton X-100 (the resulting current was $81 \pm 4\%$ of the response to GABA ($p = 0.003$)). Although flunitrazepam slightly increased the response to GABA in the presence of Triton X-100 (from $70 \pm 4\%$ to $81 \pm 4\%$ of control), this effect was not significant ($p = 0.106$). The current decay in the presence of GABA appeared to be faster when Triton X-100 and flunitrazepam were co-applied than when either of the two modulators were applied alone (Fig. 5B).

The extent of desensitization was assessed as the magnitude of the 'end current' relative to the peak current of responses to 5-min GABA applications (see Materials and methods). Triton X-100 and flunitrazepam (1 μM) both significantly reduced the ratio of end current to peak current of GABA responses, whether applied alone or in combination, indicating that both compounds increased the extent of desensitization (Fig. 5D, $p < 0.0001$). In control responses, the ratio of end current to peak current was 0.17 ± 0.01, whereas in the presence of Triton X-100, flunitrazepam, or Triton X-100 and flunitrazepam it was reduced to 0.08 ± 0.01, 0.07 ± 0.01 and 0.06 ± 0.01, respectively ($n = 6$–10). A one-way ANOVA analysis revealed no difference in end current to peak current ratio for GABA responses in the presence of Triton X-100, flunitrazepam, or the two in combination. This suggests that flunitrazepam did not further increase the extent of desensitization of responses to GABA in the presence of

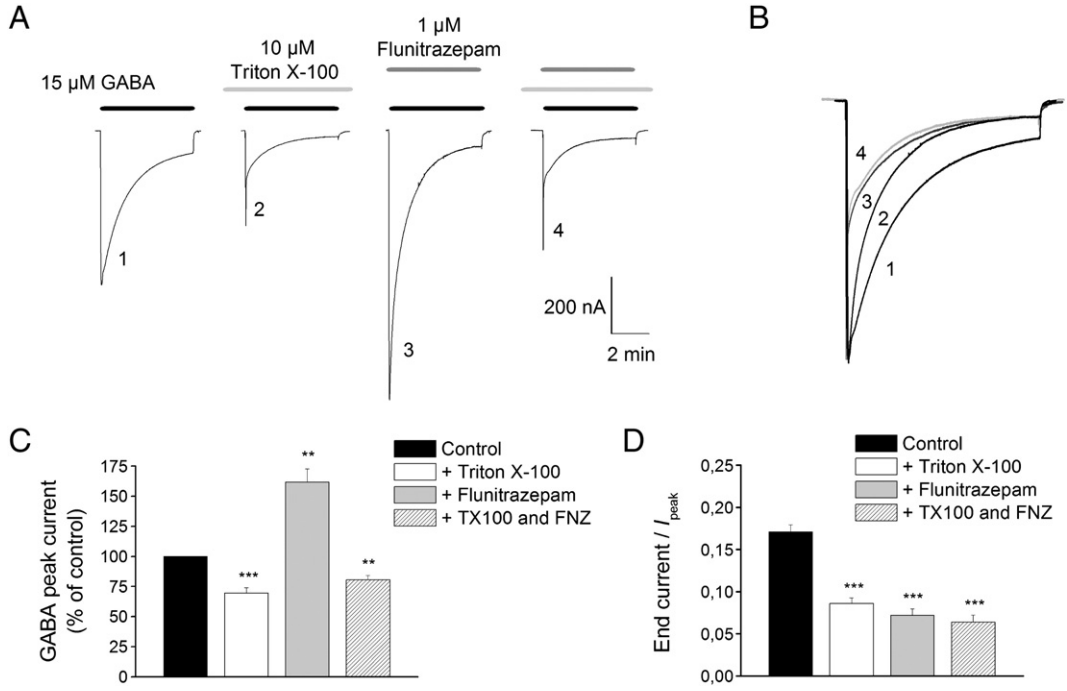

Fig. 5. Triton X-100 suppresses positive modulation of $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors by flunitrazepam. (A) Current recordings from a single oocyte at a holding potential of −60 mV showing current responses to 5-min applications of GABA (15 μM), GABA in the presence of Triton X-100 (10 μM, pre-applied for 60 s), GABA in the presence of flunitrazepam (1 μM), and GABA in the presence of Triton X-100 plus flunitrazepam. (B) The currents shown in (A) have been normalized with respect to peak current amplitude and superimposed for comparison of the current decay time courses. (C) and (D) Effects of Triton X-100 (TX100) and flunitrazepam (FNZ), applied either alone or in combination, on the peak current amplitude and the relative magnitude of the 'end current' of responses to 15 μM GABA. The results were obtained in experiments as that in (A). Peak current values were normalized to control (GABA alone) and end currents were measured at the end of the 5-min GABA applications. Asterisks denote significant difference from the control value ($n = 6$–10). The columns 'Triton X-100' and 'TX100 and FNZ' in (C) were not significantly different.

Triton X-100 (Fig. 5D). A significant increase in the extent of desensitization by the compounds was also found when this parameter was estimated from the ratio of extrapolated steady-state current to peak current (data not shown, see Materials and methods).

3.6. The effect of Triton X-100 on $\alpha_1\beta_3$ GABA$_A$ receptors

Since Triton X-100 suppressed the positive allosteric effect of flunitrazepam, which is dependent on the presence of the γ subunit [37], this might indicate that Triton X-100 uncoupled the contribution of the γ_2 subunit to the overall properties of the GABA$_A$ receptor. We therefore further examined whether the modulatory effects of Triton X-100 are dependent on the presence of a γ subunit in the GABA$_A$ receptor. In oocytes expressing $\alpha_1\beta_3$ GABA$_A$ receptors, Triton X-100 caused a small, but significant, suppression ($9 \pm 2\%$; $p = 0.005$; $n = 5$) of the peak currents evoked by 5 μM GABA and increased the apparent rate of desensitization (Fig. 6A–C). The concentration of GABA was chosen so that the relative levels of activation of $\alpha_1\beta_3$ receptors (5 μM, EC_{50} 3 μM (data not shown)) and $\alpha_1\beta_3\gamma_{2S}$ receptors (15 μM, EC_{50} 10 μM) were comparable. The inhibitory effect of Triton X-100 on peak currents was significantly stronger on receptors containing the γ_{2S} subunit (Fig. 1C (41%) and Fig. 6D (9%)).

Furthermore, Triton X-100 reduced the ratio of end current to peak current from 0.27 ± 0.01 to 0.13 ± 0.01 ($p < 0.0001$; $n = 5$) (Fig. 6D), indicating an increase in the extent of desensitization at these receptors in the presence of Triton X-100. Thus, the presence of a γ-subunit is not essential for the ability of Triton X-100 to modulate the activity of the GABA$_A$ receptor.

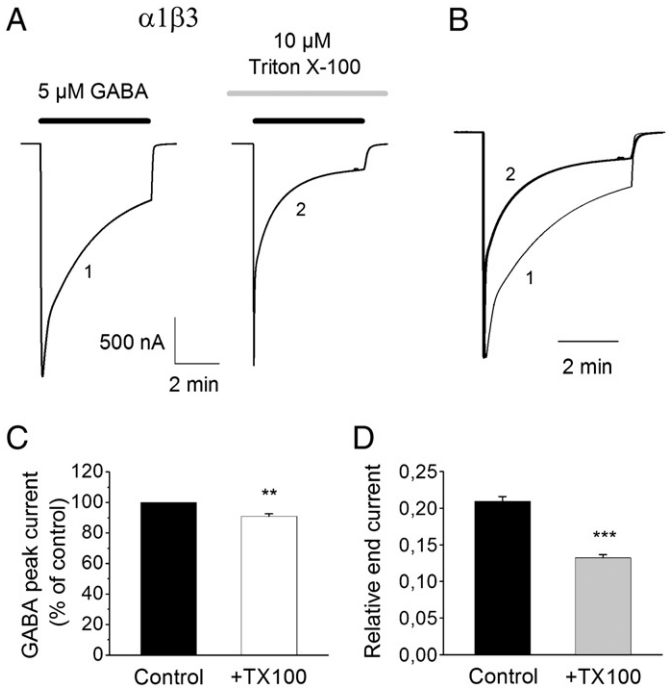

Fig. 6. The effects of Triton X-100 on GABA$_A$ receptor peak currents and apparent desensitization are not dependent on the γ subunit. (A) GABA currents in a single oocyte expressing $\alpha_1\beta_3$ GABA$_A$ receptors at a holding potential of −60 mV. The currents were evoked by 5-min applications of GABA (5 μM) in the absence or presence of Triton X-100 (10 μM), which was pre-applied for 60 s. (B) The currents shown in (A) have been normalized with respect to peak amplitude and superimposed. (C) and (D) Effects of Triton X-100 (TX100) on GABA peak current amplitude and ratio of GABA 'end' current to peak current. Data are from five experiments as that in (A). Peak currents are normalized to control. 'End currents' were measured at the end of the GABA applications. The asterisks denote significant difference from control ($p < 0.01$ (**) or $p < 0.001$ (***)).

4. Discussion

This study investigated the effects of the amphiphile Triton X-100 on currents mediated by recombinant $\alpha_1\beta_3\gamma_{2S}$ and $\alpha_1\beta_3$ GABA$_A$ receptors. The finding that Triton X-100 affects the apparent rate and extent of desensitization is in agreement with our previous data showing the ability of amphiphiles to modulate the function of GABA$_A$ $\alpha_1\beta_2\gamma_{2S}$ receptors heterologously expressed in mammalian cells [29]. However, it should be noted that the suppression of peak currents in the present study is likely to have been overestimated due to the fast current kinetics in the presence of Triton X-100. Fast kinetic events are poorly resolved in two-electrode voltage-clamp recordings in the *Xenopus* oocyte expression system; thus, a more accurate determination of fast kinetic parameters should be performed in a different experimental system. The effects of Triton X-100 could be explained by several distinct mechanisms of action. First, this amphiphilic compound may interact with the GABA$_A$ receptors in a specific pharmacological manner through binding to distinct sites on the receptors accessible from either the aqueous extracellular phase or through the lipid membrane hydrophobic interior. Second, Triton X-100 partitions into lipid bilayers and has been shown to change the elastic properties of artificial lipid bilayers and biological membranes [9,18]. It is possible that altered bilayer elastic properties could change the conformational restraint exerted by the surrounding membrane on the receptor protein, thereby resulting in a shift of the protein conformational equilibrium toward desensitized receptor states. We recently provided evidence that such a nonspecific mechanism may underlie the modulation by Triton X-100 and other amphiphiles of GABA$_A$ receptor currents in a mammalian expression system [29]. In agreement with this hypothesis, studies have provided evidence that Triton X-100 promotes inactivation of L-type Ca^{2+} and voltage-gated Na^+ channels at low micromolar concentrations through changes in lipid bilayer elastic properties [9,18]. Third, Triton X-100 might "destabilize" the GABA$_A$ receptor complex through a direct, but nonspecific, interaction with hydrophobic cavities in the protein or a disruption of important hydrophobic interactions in the transmembrane region of the protein.

Several issues emerging from this study may shed light on the mechanism(s) underlying the effects of Triton X-100 and need to be discussed in greater detail. The first aspect is the stronger inhibitory effect of Triton X-100 on GABA-evoked steady-state currents than on peak currents. This characteristic cannot be fully explained as a result of slow association with receptor binding sites accessible in the resting state as it was also observed when Triton X-100 had been pre-applied for several minutes before application of GABA. This may indicate that receptor activation facilitates interaction of Triton X-100 with an inhibitory site, e.g., a site in the channel lumen. However, repeated GABA applications in the presence of Triton X-100 did not lead to accumulated inhibition, as would be expected for an open-channel blocker unless the association rate was very fast. Furthermore, the fact that pre-incubation in Triton X-100 increases blockade of peak currents indicates that the amphiphile also interacts with closed receptor states. Additionally, a rebound current was not observed upon simultaneous removal of GABA and Triton X-100. Rebound currents are a characteristic of GABA$_A$ receptor antagonists proposed to act as open channel blockers such as penicillin, furosemide and isoflurane [38–40]. Thus, although it cannot be excluded that Triton X-100 interacts with a site in the channel pore, such a mechanism is unlikely to explain the observed inhibitory effect.

As evident from the *I–V* relationships (Fig. 3), the suppression of steady-state currents in the presence of Triton X-100 was more pronounced at positive membrane potentials, suggesting that Triton X-100 interacts with a site on the receptor within the membrane electric field. This could be in the channel pore. However, consistent with previous reports, GABA responses in the absence of Triton X-100 exhibited significant rectification, which has been suggested to be due

to a higher sensitivity to GABA at positive potentials at $\alpha_1\beta_3\gamma_{2L}$ receptors [41]. The observed voltage dependence of Triton X-100 may therefore be a consequence of the rectification rather than a direct block of the channel pore, i.e. the actions of Triton X-100 might be facilitated by receptor activation, which is higher at positive than negative potentials.

It is interesting that Triton X-100 is able to almost abolish the enhancement by flunitrazepam of GABA-evoked currents mediated by $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors. This could suggest a specific pharmacological interaction of Triton X-100 with the benzodiazepine site. However, the observed effects were independent on the presence of a γ subunit in the receptor complex, ruling out the benzodiazepine site as the molecular target for Triton X-100. At high concentrations Triton X-100 acted as a $GABA_A$ receptor agonist. A possible explanation is the existence of a second specific interaction site on the receptor complex of lower affinity than the putative inhibitory/modulatory site, as suggested for neurosteroids and barbiturates ([42,43]). However, this idea seems unlikely as it implies that the stimulatory effect of binding to such a low-affinity site can be detected in the presence of a strong, possibly maximal, stimulation of the inhibitory site. If the inhibitory actions of the amphiphile on GABA-evoked currents were mediated by a pore-blocking mechanism, Triton X-100 would be expected to exert a robust inhibitory effect at a 30-fold higher concentration. In addition, a rebound current was not observed upon removal of Triton X-100.

Although contradictory at first, both the inhibitory and stimulatory actions of Triton X-100 could be accounted for by a single, simple mechanism of action by which Triton X-100 changes bilayer elastic properties and thereby causes a shift in the conformational equilibrium of the receptor toward the open and desensitized states. The currents activated by the amphiphile at high micromolar concentrations had very small amplitudes relative to the maximal GABA response, which indicates that Triton X-100 may have a weak stabilizing effect on the agonist-unbound open state of the receptor. This putative open-state stabilization was not apparent from recordings that showed the action of Triton X-100 on *GABA-evoked* currents. However, it is plausible that such an effect would be obscured by the substantial increase in the apparent rate of current desensitization observed in the presence of Triton X-100; particularly in two-electrode voltage clamp recordings on oocytes in which peak currents are poorly resolved. This mechanism would also explain the well-established ability of Triton X-100 to increase high-affinity agonist binding to native $GABA_A$ receptors [20,23–25,27,28]. Such stabilization of open and desensitized states could be due to a direct interaction with the receptor complexes but it is also possible that the effect is mediated indirectly by changes in membrane elasticity, as we recently proposed [29]. It should be noted that the evidence that suggests that Triton X-100 increases bilayer elasticity at low micromolar concentrations is provided by measurement of its effect on gramicidin channel activity [19,44]. The possibility that amphiphilic compounds such as Triton X-100 interact specifically with gramicidin channels, e.g., at the subunit–subunit interface, cannot be excluded; however, specific interactions between amphiphiles and these simple peptide ion channels have not been identified. Thus, a strong stabilization of the desensitized receptor state(s) concomitantly with a stabilization of the open state by Triton X-100 may explain the results.

A few studies provide evidence that the modulatory effects of Triton X-100 observed in this study are not restricted to $GABA_A$ receptors but may extend to other Cys-loop receptors. A recent study shows that Triton X-100 and Tween 80, but not cholic acid, Tocrisol or DMSO noncompetitively inhibits agonist-induced nACh receptor currents without altering the potency of the agonist acetylcholine, suggesting that the receptor inhibition is not a general property of detergents [32]. Triton X-100 furthermore stabilizes reconstituted nACh receptors in an apparently desensitized state [45], supporting the hypothesis that $GABA_A$ receptors are stabilized in a similar state by Triton X-100.

It cannot be excluded that the underlying cause of the observed effects of Triton X-100 on $GABA_A$ receptor currents is a "destabilization" of the $GABA_A$ receptor protein complex through a nonspecific, but direct, interaction of the amphiphile with hydrophobic cavities or interference with structurally important interactions in the protein. This could affect the ability of the receptors to undergo the conformational changes that underlie channel opening and receptor desensitization. However, since several structurally distinct membrane proteins, such as voltage-dependent Na^+ and L-type Ca^{2+} channels and nACh receptors are affected similarly by Triton-X, this seems rather unlikely.

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