TROPISETRON MODULATION OF THE GLYCINE RECEPTOR: FEMTOMOLAR POTENTIATION AND A MOLECULAR DETERMINANT OF INHIBITION

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Abstract

The 5-hydroxytryptamine type-3 receptor antagonist, tropisetron, is in clinical use as an anti-emetic drug. This compound also exerts both potentiating and inhibitory effects on the glycine receptor chloride channel. The inhibitory effects occur at micromolar concentrations whereas the potentiating effects are shown here to occur at femtomolar concentrations at the homomeric α1 receptor. Potentiation occurred only when tropisetron was applied in the presence of glycine. This investigation also sought to identify molecular determinants of tropisetron inhibition at the α1 glycine receptor by serially mutating residues located in or near known ligand-binding sites. We discovered that conservative mutations to N102 ablated tropisetron inhibition without affecting the magnitude or sensitivity of tropisetron potentiation. Several lines of evidence, including a structure-activity analysis of tropisetron, atropine and SB203186, suggest that N102 may bind to the tropisetron tropane nitrogen via H-bonding. Mutation of the N125 residue in the β subunit, which corresponds to N102 in the α1 subunit, had little effect on tropisetron inhibitory potency. These results show that N102 is required for tropisetron inhibition but not potentiation and that inhibitory tropisetron binds in different orientations at different subunit interfaces. To our knowledge, tropisetron is the most exquisitely sensitive compound yet identified for a cys-loop ion channel receptor.

Keywords: ligand-gated ion channel; cys-loop receptor; 5-HT_3 receptor antagonist; tropeine; femtomolar; binding site; molecular structure and function.

Running title: Tropisetron modulation of the glycine receptor

Introduction

Glycine receptor (GlyR) Cl^- channels mediate inhibitory neurotransmission at defined synapses in the spinal cord, brainstem, cerebellum and retina (Legendre 2001; Lynch 2004). In addition, extrasynaptic GlyRs are widely distributed throughout the central nervous system (e.g., Flint et al. 1998; McCool and Farroni 2001). GlyRs are members of the cys-loop family of ligand-gated ion channel receptors which also includes cation-permeable nicotinic acetylcholine receptors (nAChRs), 5-hydroxytryptamine type-3 receptors (5-HT_3Rs) and anion-permeable GABA type-A receptors ($GABA_A$Rs). Functional cys-loop receptors comprise an assembly of 5 homologous membrane-spanning subunits arranged symmetrically around a central water-filled pore (Unwin 2005). The GlyR can be formed either as a homomer of α subunits or as a heteromer of α and β subunits in an invariant 2α:3β stoichiometry (Griffon et al. 1999; Grudzinska et al. 2005). All cys-loop receptor subunits consist of a large extracellular ligand-binding domain followed by a transmembrane domain that comprises a bundle of 4 α-helices. Ligand-binding pockets are located at the subunit interfaces approximately midway between the top and bottom of the ligand-binding domains (Brejc et al. 2001). This pocket is lined by 3 loops (labelled A, B and C) from one subunit that form the 'principal' (or +) side of the ligand-binding pocket, whereas 3 β-strands (labelled D, E and F) from the adjacent subunit form the 'complementary' (or -) side.

GlyRs have recently emerged as potential therapeutic targets for anti-spasticity and anti-inflammatory analgesic drugs (Laube et al. 2002; Zeilhofer 2005; Lynch and Callister 2006). Their involvement in the regulation of motor control via local spinal reflex circuits is dramatically illustrated by the motor deficits that result when glycinergic current is reduced in the human hereditary disorder, hyperekplexia (Bakker et al. 2006). This disorder is characterized by an exaggerated reflex response to unexpected stimuli, which typically takes the form of a complete but temporary muscular rigidity, leading to a loss of balance and an unprotected fall. A role for GlyRs in inflammatory pain sensitization is implied by the observation that prostaglandin E2 mediates inhibition of α3 subunit-containing GlyRs in dorsal horn nociceptive neurons (Ahmadi et al. 2002; Harvey et al. 2004). This process is likely to result in the disinihibition of firing activity in nociceptive projection neurons, thereby increasing the transmission of nociceptive stimuli to the brain (Zeilhofer 2005; Lynch and

Callister 2006). Together, these observations imply that compounds that potentiate (i.e., restore) GlyR current flux may be useful as leads for novel analgesic or muscle relaxant drugs.

Orally-administered tropisetron is used clinically for treating postoperative and chemotherapy-induced emesis (Haus et al. 2004). Its therapeutic effects are mediated via antagonism of the 5-HT_3R, which is strongly expressed in medullary emesis control centres (Farber et al. 2004). However, tropisetron also has potent effects on the GlyR. It has been shown to potentiate glycine currents at nanomolar concentrations, but produce inhibition at higher (micromolar) concentrations (Chesnoy-Marchais 1996; Supplisson and Chesnoy-Marchais 2000). The high potency, strong potentiating effects and excellent oral bioavailability of tropisetron render it suitable as a therapeutic lead compound for the development of novel GlyR-specific drugs. However, a significant problem with existing tropeine derivatives is that they are not specific for the GlyR (Maksay et al. 2004). Although structure-activity analyses of tropisetron analogues have provided insight into the tropisetron structural moieties required for potentiation and inhibition (Chesnoy-Marchais 1996; Maksay 1998; Chesnoy-Marchais 1999; Chesnoy-Marchais et al. 2000; Maksay et al. 2004), there are as yet few clues as to the location or structure of the GlyR tropisetron binding sites.

As the 5-HT_3R and GlyR are both members of the cys-loop receptor family, it is reasonable to hypothesise that they may share common or overlapping tropisetron binding sites. Although little is known about tropisetron binding at the 5-HT_3R, recent studies have produced a consensus model for the binding of the structurally-related compound, granisetron, in the agonist-binding pocket (Maksay et al. 2003; Thompson et al. 2005; Yan and White 2005). It should be noted, however, that granisetron binds to the α1 5-HT_3R with nanomolar affinity (Schreiter et al. 2003; Thompson et al. 2005; Yan and White 2005), whereas it inhibits the α1 GlyR with an IC_{50} of around 100 μM (Chesnoy-Marchais et al. 2000). Furthermore, it is not known whether the inhibitory or potentiating tropisetron-binding sites of the GlyR overlap with each other or whether either of them lie in the agonist-binding pocket. Thus, the extent to which the 5-HT_3R granisetron binding site provides a useful template for modelling the structure of either the potentiating or inhibitory GlyR tropisetron binding sites remains to be seen.

To understand the molecular interactions crucial for high potency tropisetron potentiation, it is first necessary to characterise the molecular determinants of both its inhibitory and potentiating effects. The present study identifies a specific molecular determinant of tropisetron inhibition. During the course of these experiments, we also found that tropisetron potentiates the GlyR at surprisingly low concentrations.

Materials and Methods

GlyR mutagenesis and expression

The human GlyR α1 subunit cDNA was subcloned into the pCIS2 plasmid vector. The human β subunit was subcloned into the pIRES2-EGFP plasmid vector (Clontech, Palo Alto, CA, USA). Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) and the successful incorporation of mutations was confirmed by sequencing the clones. HEK293 cells were transfected using a calcium phosphate precipitation protocol. When co-transfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1:20. After exposure to transfection solution for 24 hrs, cells were washed twice using phosphate buffered saline and used for recording over the following 24 - 72 hrs.

Electrophysiology

Cells were observed using a fluorescent microscope and currents were measured by whole cell recording. Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark). Pipettes had tip resistances of 1.5 - 3 MΩ when filled with pipette solution containing (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. Cells were voltage-clamped at –40 mV at room temperature (19 – 22 ^{o}C) and membrane currents were recorded using an Axopatch 1D amplifier and pCLAMP9.2 software (Axon Instruments, Union City, CA, USA). GFP fluorescence was used to identify cells expressing the GlyR β subunit. The successful incorporation of

β subunits into functional receptors was also determined by their reduced picrotoxin sensitivity (Pribilla et al. 1992), as discussed below.

Picrotoxin (Sigma, St Louis, MO) was stored frozen as a 100 mM stock in dimethylsulfoxide. Tropisetron (Sigma), atropine (Sigma) and SB 203186 (Alexis Biochemicals) were made as 100 mM stocks in water and stored at 4 ^{o}C for up to 2 weeks.

Solution exchanges were performed using a parallel array of microtubular barrels through which solutions were gravity-fed into the recording chamber. As the solution flow rate was typically 1 ml.min^{-1} and the microtubules had an internal diameter of 300 μm, the flow velocity was around 0.2 m.s^{-1}. Barrel mouths were positioned to within 500 μm of target cells and rapid complete solution exchange was effected by moving the barrels laterally under the control of a manual micromanipulator. Solution exchange time constants of 100 ms were routinely achieved between adjacently placed barrels. Cells, cultured on glass coverslips, were placed in the recording chamber. Given the extreme sensitivity of the GlyRs to tropisetron, we assumed that all unpatched cells on a coverslip were contaminated after a single tropisetron application to a single cell. For this reason, we recorded from only one cell per coverslip and the recording chamber was thoroughly rinsed with control solution before a new coverslip was introduced.

Data analysis

Results are expressed as mean ± standard error of the mean of 3 or more independent experiments. The Hill equation was used to calculate the saturating current magnitude (I_{max}), half-maximal concentration (EC_{50}) and Hill coefficient (n_H) values for glycine activation. A similar equation was also used to calculate the half maximal concentrations for tropisetron potentiation (EC_{50}) and inhibition (IC_{50}). Inhibitory dose-responses were quantitated relative to the maximum potentiation. All curves were fitted using a non-linear least squares algorithm (Sigmaplot 9.0, Jandel Scientific, San Rafael, CA, USA). Statistical significance was determined by paired or unpaired Student's t-test, as appropriate, with $P < 0.05$ representing significance.

Results

Tropisetron modulation of homomeric α1 GlyRs

Sample glycine dose-response relationships for α1 and α1β GlyRs are shown in Fig. 1A. The averaged dose-response curves are presented in Fig. 1B, with mean parameters of best fit displayed in Table 1. The incorporation of β subunits into heteromeric α1β GlyRs results in a characteristic reduction in receptor sensitivity to picrotoxin (Pribilla et al. 1992). To confirm the successful incorporation of β subunits, the effects of 10 μM picrotoxin were measured in the presence of an EC_{30} glycine concentration at both the α1 and α1β GlyRs. Examples of the effects of 10 μM picrotoxin on putative homomeric and heteromeric receptors are shown in Fig. 1C. The percentage of original current remaining in 10 μM picrotoxin, averaged from 4 cells expressing each receptor, is presented in Table 1. The results show that co-expression of α1 and β subunits indeed results in the formation of heteromeric receptors. Fig. 1 also describes the glycine and picrotoxin sensitivities of 3 mutant receptors which will be considered below.

We were surprised to find that femtomolar concentrations of tropisetron caused significant potentiation of submaximal glycine responses in the α1 GlyR. The biophysical implications of such high sensitivity are considered in the Discussion. Briefly, the unbinding rate should be so slow that binding is essentially irreversible and thus, the time course of tropisetron potentiation should provide a reasonable estimate of the tropisetron binding rate (k_{on}). To investigate this rate, we quantitated the time course of the potentiation induced by 1 pM tropisetron. A sample experiment is shown in Fig. 2A, with the tropisetron-mediated current change plotted against the tropisetron exposure time in Fig. 2B. This plot was fitted with a time constant of 39 s. The time constant averaged from 4 similar experiments was 107 ± 26 s. As considered in detail below, this value corresponds well with the theoretical maximum diffusion limited rate of 10^9 M^{-1} s^{-1}. As shown in Fig. 2A (lower panel), a 100 s application of 1 pM tropisetron in the absence of glycine did not significantly potentiate a subsequent response to glycine. Similar results were seen in each of 8 cells.

In continuous electrophysiological recordings, we found that tropisetron potentiation showed no significant reversal for periods of up to 30 min after washout. To test its reversibility over a longer (5 hr) period, we incubated cells in petri dishes for 5 min in control bath solution containing 10 fM

tropisetron plus 20 μM (EC_{30}) glycine. We then removed the tropisetron from the cells by thorough washing, and incubated the cells in standard culture medium for 5 hrs. We then tested whether GlyRs could be potentiated by 1 pM tropisetron. In a total of 4 cells investigated in this manner, we found that 20 μM glycine produced almost saturating (EC_{90}) glycine current suggestive of strong tropisetron potentiation. Furthermore, 1 pM tropisetron had no significant effect on any of the 4 cells. This indicates that tropisetron potentiation is completely irreversible over a 5 hr period.

Tropisetron potentiating and inhibitory dose-response relationships were measured in the presence of a 20 μM (EC_{30}) glycine concentration. Because the effects of tropisetron were irreversible, we measured dose-response relationships by applying progressively higher tropisetron concentrations at approximately 3 minute intervals. We recognise that this was unlikely to be sufficient time for tropisetron to equilibrate with its site, and thus our results may be subject to a systematic error. Nevertheless, we considered that this approach should provide an order of magnitude approximation and should also be useful for assessing the effects of mutagenesis on the sensitivity of tropisetron potentiation. Samples of the potentiating response of the α1 GlyR to increasing concentrations of tropisetron in the femtomolar range are shown in Fig. 2B (upper panel) and results averaged from 5 cells are presented in Fig. 2C (lower panel). The data were fitted with mean EC_{50} and n_H values of 4.4 ± 2.5 fM and 0.7 ± 0.2, respectively (both n = 5). Tropisetron induced a maximum potentiation of 83 ± 10 % (n = 5) of the EC_{30} glycine current. The tropisetron inhibitory potency was measured in a similar manner. Examples of EC_{30} glycine currents recorded in the presence of inhibitory (micromolar) concentrations of tropisetron are shown in Fig. 2C (upper panel), with results averaged from 7 cells presented in Fig. 2C (lower panel). The parameters of best fit for tropisetron inhibition are shown in Table 1.

The glycine-dependence of tropisetron potentiation and inhibition was determined. Fig. 3A (left panel) shows examples of the effects of a maximally potentiating (10 nM) concentration of tropisetron on currents activated by EC_{30}, EC_{70} and EC_{90} glycine in different cells expressing α1 GlyRs. Results averaged from 5 cells at each glycine concentration reveal that the percentage current increase declines as glycine concentration increases (Fig. 3A, right panel). A previous study noted a generally similar phenomenon, with the exception that no potentiation was observed at glycine

concentrations greater than EC_{30} (Supplisson and Chesnoy-Marchais 2000). Fig. 3B (left panel) shows examples of the inhibitory effect of 200 μM tropisetron on currents activated by EC_{30}, EC_{70} and EC_{100} glycine in a cell expressing homomeric α1 GlyRs. Results of similar experiments averaged from 4 cells confirm that tropisetron inhibition is progressively overcome by increasing the glycine concentration (Fig. 3B, right panel). As discussed in detail below, this result is consistent with tropisetron acting as a competitive antagonist.

A molecular determinant of tropisetron inhibition

We hypothesised that the inhibitory tropisetron-binding site coincides with one of the known ligand-binding sites of the GlyR. Accordingly, we screened tropisetron against α1 GlyRs containing mutations to a variety of known or suspected ligand-binding sites. We investigated the principal ligand-binding domain A (via mutations A101C and N102A), principal ligand-binding domain B (via F159A and Y161C), principal ligand-binding domain C (via K200A, H201A, Y202F, N203A, T204A and Y207A) and complementary ligand-binding domain D (via F63Yand R65A). All of these residues have previously been identified as either glycine binding residues or as residues that lie adjacent to glycine binding residues (Vandenberg et al. 1992b; Vandenberg et al. 1992a; Schmieden et al. 1993; Rajendra et al. 1995; Vafa et al. 1999; Han et al. 2001; Grudzinska et al. 2005). By inspection of a molecular model of the GlyR extracellular domain (Nevin et al. 2003), we identified and tested several other potential ligand-binding residues in and around the ligand-binding pocket via the N46Q, S129V, A137S, I153T and D180E mutations. We also tested the alcohol-binding site (via S267C) (Mihic et al. 1997) and the inhibitory zinc-binding site (via H107N and H109N) (Harvey et al. 1999; Nevin et al. 2003). Residues homologous to F63, R65, H101, D180, K200 and Y207 in the GlyR α1 subunit have been proposed to contribute to the granisetron-binding site in the 5-HT_3R (Maksay et al. 2003; Thompson et al. 2005; Yan and White 2005). Many of the residues investigated here are displayed in the model in Fig. 4.

With the exception of Y207A, we obtained robust glycine-gated currents upon recombinant expression of homomeric α1 GlyRs incorporating any of the above mutations. Glycine concentrations of up to 50 mM failed to significantly activate the $\alpha1^{Y207A}$ GlyR, which may not be surprising given its role as a glycine binding site (Grudzinska et al. 2005). All mutant GlyRs were screened at a strongly inhibiting (500 μM)

tropisetron concentration in the presence of an EC_{30} glycine concentration. Results averaged from at least 4 cells expressing each mutant GlyR, together with the EC_{30} glycine concentration required to activate each receptor, are listed in Table 2. Of all the mutants tested, only the N102A mutation resulted in the complete elimination of tropisetron inhibition, to the effect that 500 μM tropisetron induced a strong potentiation.

This finding prompted us to test the effects of the more conservative substitutions, N102D and N102Q, on tropisetron inhibitory potency. As summarised in Table 2, all three N102 mutations acted similarly to abolish inhibition without significantly affecting the maximum tropisetron potentiating amplitude. Because N102Q is the most conservative of these substitutions, we analysed the $\alpha1^{N102Q}$ GlyR in more detail. Examples of currents recorded in the presence of increasing glycine concentrations in one cell are shown in Fig. 1A, with the dose-response curve averaged from 7 cells presented in Fig. 1B. The mean glycine EC_{50} and n_H values are summarised in Table 1. The glycine EC_{50} was increased by about an order of magnitude relative to the wild type α1 GlyR, consistent with the role of N102 as a glycine binding site (Vafa et al. 1999). The N102Q mutation had little effect on the picrotoxin sensitivity of α1 GlyRs (Fig. 1C, D). As the tropisetron potentiation dose-responses were measured as described above for the α1 GlyR, the results are directly comparable. Examples of the effects of increasing tropisetron concentrations on currents activated by 220 μM (EC_{30}) glycine are shown in Fig. 5A with the averaged dose-response curves shown in Fig. 5B. Tropisetron potentiated the $\alpha1^{N102Q}$ GlyR with mean EC_{50} and n_H values of 5.3 ± 2.0 fM and 1.0 ± 0.3, respectively (both n = 5), with a maximum potentiation of 65 ± 5 % (n = 5) of the EC_{30} glycine current. As none of these values differed significantly from the respective α1 GlyR values, we conclude that N102Q has no effect on the tropisetron potentiation binding site.

The role of the β subunit in tropisetron inhibition

The β subunit residue, N125, is homologous with the α1 subunit N102 residue. To investigate whether N125 is also a determinant of tropisetron inhibition, we compared the inhibitory potencies of tropisetron at recombinantly expressed $\alpha1\beta$, $\alpha1^{N102Q}\beta$ and $\alpha1^{N102Q}\beta^{N125D}$ GlyRs. Because αβ GlyR heteromers exhibit a drastically reduced picrotoxin sensitivity relative to α homomers (Pribilla et al.

1992), we first confirmed the presence of β subunits by measuring the picrotoxin sensitivity of all heteromeric GlyRs. Sample current responses to increasing concentrations of glycine for all 3 heteromeric GlyRs are shown in Fig. 1A. The averaged glycine dose-response curves shown in Fig. 1B, with the mean parameters of best fit summarised in Table 1. Examples of the response of the same GlyRs to 10 μM picrotoxin are shown in Fig. 1C. Because the averaged picrotoxin inhibitory responses were significantly reduced compared to those of the respective homomers (Fig. 1D), we concluded that β subunits were successfully incorporated into at least the majority of functionally expressed GlyRs.

Examples of the effects of increasing concentrations of tropisetron on currents activated by EC_{30} glycine in cells expressing recombinant α1β, $\alpha1^{N102Q}$, $\alpha1^{N102Q}\beta$ and $\alpha1^{N102Q}\beta^{N125D}$ GlyRs are shown in Fig. 6A. Averaged dose-response curves are presented in Fig. 6B, with the corresponding curve for the α1 GlyR included as a dashed line for comparison. All averaged curve fit parameters are summarised in Table 1. The $\alpha1^{N102Q}\beta$ and α1β GlyRs exhibited comparable tropisetron IC_{50} values (Fig. 6B, Table 1), implying the presence of tropisetron inhibitory sites on the β subunit. The $\alpha1^{N102Q}\beta^{N125D}$ GlyR exhibited only a minor reduction in tropisetron inhibitory potency relative to that of the $\alpha1^{N102Q}\beta$ GlyR (Fig. 6B, Table 1). This implies that the location of the β subunit inhibitory tropisetron site is not identical to its location on the α1 subunit. Finally, it is apparent that co-expression of the β subunit significantly increased the mean IC_{50} and n_H values for tropisetron inhibition (Fig. 6A, Table 1), indicating positive cooperativity among β subunit tropisetron inhibitory sites.

Structure-activity relationships of tropisetron analogues

To identify the tropisetron moiety likely to interact with N102, we compared the inhibitory potencies of tropisetron, atropine and SB 203186 at the α1 and $\alpha1^{N102Q}$ GlyRs. As shown in Fig. 7A, SB 203186 replaces the tropisetron tropane group but retains the indole rings intact. In contrast, atropine retains the tropane group but disrupts the indole structure. Examples of the effects of increasing concentrations of atropine and SB 203186 on the α1 GlyR are shown in Fig. 7B. The α1

GlyR atropine dose-response, averaged from 3 cells, is shown in Fig. 7C (filled circles) and reveals mean IC_{50} and n_H values of 234 ± 24 μM and 2.0 ± 0.1 (both n = 3), respectively. These are comparable with the values reported previously for atropine at these receptors (Maksay et al. 1999). Like tropisetron, atropine did not significantly inhibit the $\alpha1^{N102Q}$ GlyR at concentrations up to 500 μM (Fig. 7C). SB 203186 elicited a strong potentiation (Fig. 7B) but had no significant inhibitory effect on the α1 GlyR at concentrations up to 150 μM (n = 10). These results indicate the tropisetron tropane group is essential for inhibition.

Discussion

Comparison with previous results

We were surprised to find that tropisetron potentiated the α1 GlyR at femtomolar concentrations. We observed significant tropisetron potentiation at EC_{30} and EC_{70} glycine concentrations but none at a near saturating glycine concentration (Fig. 3A). We also found that tropisetron inhibited currents activated by EC_{30} glycine with an IC_{50} near 90 μM, and that this inhibition could be overcome by increasing the glycine concentration (Fig. 3B). These results contrast in 3 respects with those of a previous electrophysiological study conducted on α1 GlyRs recombinantly expressed in *Xenopus* oocytes (Supplisson and Chesnoy-Marchais 2000). First, Supplisson and Chesnoy-Marchais reported a tropisetron EC_{50} of around 5 μM for α1 GlyR homomers, although incorporation of the β subunit reduced the EC_{50} to the low nanomolar range. Second, they reported that tropisetron potentiation was apparent only at glycine concentrations of less than EC_{30}. Third, contrary to the present study they found that the tropisetron inhibitory potency was enhanced with increased glycine concentration. As both studies examined the same recombinantly expressed GlyR subunits, these differences most likely arise as a consequence of the different expression systems used (*Xenopus* oocytes versus HEK293 cells).

The effects of tropisetron have also been investigated on native GlyRs in embryonic spinal neurons (Chesnoy, 1996). Although the tropisetron EC_{50} was not quantitated, a robust potentiation was seen at 20 nM. Another study showed that tropisetron displaced [^{3}H]strychnine from spinal neuron GlyRs at micromolar concentrations, although an additional nanomolar affinity displacement component was observed in the presence of glycine (Maksay, 1998). This nanomolar component was associated only with those tropeine derivatives that elicited a potentiating effect. However, it is difficult to relate these findings to the present study, as the potency with which tropisetron displaces strychnine in the presence of glycine may be different to the potency with which it potentiates glycine-activated currents. However, the binding data do support the present conclusion that tropisetron behaves as a competitive antagonist at micromolar concentrations. The structurally-related compound, atropine, was also found to act as a competitive antagonist of the α1 GlyR (Maksay et al. 1999).

Biophysical implications of femtomolar potency

Femtomolar affinity implies a very fast association rate, a very slow dissociation rate and multiple close contact points between the bound molecule and its site (Radic et al. 2005). Substances that bind with such affinity are comparatively rare in biology, although well documented examples exist in the case of biotin with streptavidin (Howarth et al. 2006), trifluoroacetophenones with acetylcholinesterase (Senapati et al. 2005; Radic et al. 2005) and zinc with metalloregulatory proteins (Outten and O'Halloran 2001). The tropisetron binding or associaiton rate, k_{on}, is unlikely to exceed the rate of diffusion. In free solution, the relationship between the k_{on} and the rate of diffusion is given by the Smoluchowski equation, $k_{on} = (4 \times \pi \times N \times D \times r)/1000$ where N is Avogadro's number, D is the diffusion coefficient and r is the radius of the target on the receptor. Using this equation, Burgen estimated an upper k_{on} limit of 2.5×10^9 M^{-1} s^{-1} for drug-receptor interactions (Burgen, 1966). This is comparable to the diffusion limited rate ($3.6 – 8.0 \times 10^9$ M^{-1} s^{-1}) with which trimethylammonio triacetophenone associates with acetylcholinesterase (Radic et al. 2005).

However, association rates for even the most energetically favourable binding interactions seldom approach the diffusion limited rate. Most femtomolar binding sites lie in deep pockets, which means that steric hindrance by protruding structures is likely to retard binding rates. Furthermore, once a drug closely approaches its receptor, intermolecular forces may either repel the molecule or attract it to lower affinity binding conformations. Together, these mechanisms can strongly reduce k_{on}.

The relationship between EC_{50} and binding affinity (K_A) is given by $EC_{50} = K_A/(1+E)$ where E is efficacy (Colquhoun, 1998). If we assume that tropisetron – GlyR interaction has low value of E (so that $EC_{50} = K_A$) and a fast k_{on} of 10^9 M^{-1} s^{-1}, then its dissociation rate will be 10^{-5} s^{-1} at an EC_{50} of 10 fM. This implies that tropisetron potentiation is essentially irreversible. It also implies that the equilibrium association time constant in the presence of 1 pM tropisetron is 1000 s. This does not agree well with the value of 107 ± 25 s observed in this study. Alternately, the E value of the system may be high, so that the tropisetron K_A is much larger than its EC_{50}. This would imply an extremely fast tropisetron-mediated receptor conformational change, but would yield a lower k_{on}. A slow dissociation rate is expected in this case as well.

A high 'apparent affinity' can result from a lipophilic ligand accumulating in the plasma membrane, thereby creating a high local concentration near a transmembrane binding site (Lee, 2004). It is unlikely that such a mechanism applies in the present case as tropisetron must be co-applied with glycine in order to effect potentiation (Fig. 2A).

At least two sources of error are likely to become significant when applying femtomolar ligand concentrations to receptors. The first stems from the fact that the ligand concentration may be close to or lower than the receptor concentration, which could result in the depletion of the local ligand concentration but this effect is likely to be minimal as ligand is applied under continuous flow, thereby maintaining constant ligand concentration. The second relates to the fact that low ligand concentrations equilibrate very slowly with the receptor, which makes it difficult to accurately measure concentration-response relationships. For these reasons, the tropisetron EC_{50} values measured in this study must be considered as order-of-magnitude approximations.

What can explain the drastic difference in tropisetron sensitivity between HEK293 cell and *Xenopus* oocyte expression systems? The glycinergic agonists, glycine, β-alanine and taurine all exhibit approximately 10-fold differences in their EC_{50} values depending on whether GlyRs are expressed HEK293 cells or *Xenopus* oocytes (Lynch 2004). The minor structural changes that mediate these differences could have a drastic effect on the affinity of a compound that binds with femtomolar affinity.

N102 as a molecular determinant of tropisetron inhibition

The conservative α1 subunit mutation, N102Q, completely abolished tropisetron inhibition of α1 homomeric GlyRs although it had no effect on the magnitude or sensitivity of tropisetron potentiation. We therefore conclude that N102 is a specific determinant of tropisetron inhibition. Several arguments suggest this residue may be an inhibitory tropisetron contact site. First, N102 has previously been identified as a glycine contact site (Vafa et al. 1999; Han et al. 2001), implying its availability to coordinate other ligands in the binding pocket. Furthermore, because N102 mutations do not affect strychnine affinity (Vafa et al. 1999) or tropisetron potentiation (this study), they are

unlikely to act by non-specifically disrupting the structure of the ligand-binding pocket. Finally, an interaction between N102 and tropisetron is chemically plausible. Because asparagine amine groups serve as H bond donors, N102 could conceivably form an H bond with the tropisetron tropane nitrogen. A number of observations suggest that such an interaction may be realistic. Selective substitution of the asparagine amine group with an oxygen, via the N102D mutation, abolishes tropisetron inhibitory potency. A comparison of the structure-activity relationships of tropisetron, SB 203186 and atropine indicates that the tropane group is essential for inhibition. Docking models of granisetron at the 5-HT_3R, which place the granisetron tropane nitrogen and the near-homologous 5-HT_3R asparagine (N128) into close physical proximity, support the steric feasibility of this proposed interaction (Maksay et al. 2003; Thompson et al. 2005). Finally, a common binding site for tropisetron and glycine fits well with functional data showing tropisetron and its analogues acting as competitive antagonists (this study; Maksay 1998; Maksay et al. 1999).

Although the tropisetron tropane nitrogen is also essential for potentiation, (Maksay 1998; Chesnoy-Marchais et al. 2000), the results of the present study indicate that N102 does not coordinate tropisetron when it binds in the potentiating mode.

The role of the β subunit

Since heteromeric GlyRs have a 2α:3β subunit stoichiometry (Grudzinska et al. 2005), they contain α–β, β–β and β–α interfaces but probably not α–α interfaces. The present study demonstrates that the α1 subunit N102Q mutation ablates tropisetron inhibitory binding at the α–α interface. However, since the β subunit N125D mutation does not affect tropisetron inhibitory potency in heteromeric $\alpha1^{N102Q}\beta$ receptors, we conclude that tropisetron binds in a different orientation at the β–β, α–β and/or β-α interfaces than it does at the α–α interface. Since the tropisetron inhibitory n_H is significantly increased in β subunit-containing GlyRs (Table 1), positive cooperativity may exist between the β subunit sites.

Conclusion

This study demonstrates that tropisetron potentiates the α1 GlyR at femtomolar concentrations. As potentiation is observed only when tropisetron is co-applied with glycine, it is unlikely to be mediated by tropisetron accumulation in the cell membrane. Our results are consistent with a model where tropisetron binds to its potentiating site at a rate that approaches the rate of diffusion and becomes coordinated so tightly that it cannot dissociate.

We have also shown that conservative mutations to N102 in the α1 subunit specifically ablate tropisetron inhibition without affecting the magnitude or sensitivity of tropisetron potentiation. We tentatively suggest that N102 may directly coordinate inhibitory tropisetron via an H bond with its tropane nitrogen. As N102 is in close proximity to the glycine-binding site, this would provide a ready explanation for the competitive nature of tropisetron inhibition. Because mutation of the homologous β subunit residue, N125, does not affect tropisetron inhibitory potency in $\alpha1^{N102Q}\beta$ GlyRs, we propose that the tropisetron inhibitory binding site may vary from one subunit interface type to another in heteromeric GlyRs. Finally, the results presented here provide a first step in defining the GlyR binding sites for a compound that is probably the most promising therapeutic lead yet identified for the GlyR.

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Table 1. Functional properties of GlyRs employed in this study.

GlyR	Glycine			Tropisetron		
	EC_{50} (μM)	n_H	n	IC_{50} (μM)	n_H	n
α1	35 ± 2	2.5 ± 0.2	10	89±13	-0.9±0.3	7
α1β	51 ± 9	1.6 ± 0.2	6	255± 40**	-2.8± 0.4***	4
$\alpha1^{N102Q}$	362±36***	2.2 ± 0.1	7	no inhibition at 500 μM		8
$\alpha1^{N102Q}\beta$	133 ± 6***	2.0 ± 0.2	6	275±54**	-2.2±0.5**	6
$\alpha1^{N102Q}\beta^{N125D}$	295 ± 36***	2.2 ± 0.6	4	384±34***	-2.9±0.4***	5

*P < 0.05, ** P < 0.01, *** P < 0.001 relative to the α1 GlyR using Student's unpaired t-test.

Table 2. Sensitivity of mutant α1 GlyRs to tropisetron inhibition. Mutations that completely abolished inhibition, while leaving potentiation intact, are shown in bold. All results were averaged from at least 4 cells.

α1 GlyR mutation	[glycine] (μM)*	% inhibition at 500 μM tropisetron
wild type	20	-76 ± 12
N46Q	25	-28 ± 4
F63Y	60	-30 ±13
R65K	5000	-70 ± 17
I93A	600	-58 ± 5
A101C	10	-49 ± 7
N102A	**800**	**+32 ± 10**
N102D	**30**	**+ 87 ± 9**
N102Q	**220**	**+43 ± 8**
H107N	25	-55 ± 11
H109N	25	-60 ± 5
S129V	12,000	-79 ± 8
A137S	30	-54 ± 6
I153T	20	-42 ± 12
F159Y	2,000	-52 ± 5
Y161A	200	-47 ± 2
D180E	25	-41± 6
K200A	700	-68±10
H201A	30	-84 ± 10
Y202F	8,000	-64 ± 3
N203A	30	-64 ± 10
T204A	10,000	-48 ± 12
S267C	25	-48 ± 8

* This indicates the EC_{30} glycine concentration used to assay the tropisetron inhibitory potency.

Figure Legends

Figure 1. Glycine and picrotoxin sensitivities of GlyRs employed in this study. A. Examples of currents activated by the indicated glycine concentrations in cells expressing the indicated GlyR subunits. In this and all subsequent figures, inward currents are represented as downward deflections and glycine was applied for the period represented by the unfilled bar. The horizontal 5 s scale bar applies to all traces displayed in this figure. B. Averaged dose-responses for all GlyRs examined in this study. Mean parameters of best fit are given in Table 1. Symbols presented in the legend apply to panels B and D. C. Examples of the inhibitory effects of 10 μM picrotoxin on currents activated by EC_{30} glycine in cells expressing the indicated GlyRs. Picrotoxin was applied for the period indicated by the filled bar. D. Averaged inhibition produced by 10 μM picrotoxin at each of the 5 tested GlyRs. A Student's unpaired t-test was used to assess statistical significance, with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ representing significance relative to the α1 GlyR.

Figure 2. Tropisetron effects on the α1 GlyR. In this and all subsequent figures, tropisetron application is indicated by the filled bars. A. (Upper panel) A continuous recording showing the effect on glycine current magnitude of repeated 10 s applications of 1 pM tropisetron. Periodic applications of saturating (2 mM) glycine reveal that the saturating current magnitude remains constant. The lower panel shows a similar experiment except that cells were exposed to a single 90 s application of 1 pM tropisetron in the closed state. B. Plot of current magnitude versus cumulative exposure time to 1 pM tropisetron for the data shown in A (upper panel). The curve is an exponential fit with a time constant of 39 s. C. A sample potentiating dose-response (upper panel) and averaged potentiating dose-responses from 5 cells expressing α1 GlyRs (lower panel). The vertical axis is plotted as $(I - I_{EC30})/I_{max}$, where I_{EC30} is the control current in absence of tropisetron, I is the current in the presence of a given concentration of tropisetron and I_{max} is the maximum amplitude of the tropisetron-potentiated current. Curve fit parameters of best fit are displayed in the text. D. A sample inhibiting dose-response (upper panel) and averaged inhibitory dose-responses from 5 cells expressing α1 GlyRs (lower panel). I_{max} is the maximum amplitude of the tropisetron-potentiated current. Curve fit parameters of best fit are displayed in Table 1.

Figure 3. Glycine dose-dependence of tropisetron potentiation and inhibition. A. (Left panel) Examples of currents activated by EC_{30}, EC_{70} and EC_{90} glycine concentrations in different cells expressing α1 GlyRs, together with the effect of a co-application of 10 nM tropisetron. The right panel shows the mean percentage tropisetron potentiation averaged from 5 cells at each glycine concentration. A Student's unpaired t-test was used to assess statistical significance, with ** $P < 0.01$ representing significance relative to the α1 GlyR. B. (Left panel) Examples of currents activated by EC_{30}, EC_{70} and EC_{100} glycine concentrations in a cell expressing α1 GlyRs, together with the effect of a co-application of 200 μM tropisetron. The right panel shows the percentage of current remaining averaged from 5 cells at each glycine concentration. A Student's paired t-test was used to assess statistical significance, with *** $P < 0.001$ representing significance relative to the α1 GlyR.

Figure 4. Model of the extracellular domain of the homomeric α1 GlyR built by homology with the AChBP structure previously as described (Nevin et al. 2003). Two of the five subunits are displayed as ribbons coloured by subunit. The model is viewed parallel to the membrane showing the outer surface of the pentameric ring, with the membrane at the bottom of the figure. The putative agonist-binding site is situated behind the orange overlapping loop at the centre of the figure. A selection of residues that have been mutated in this study are shown as bonds, coloured by atom.

Figure 5. Tropisetron potentiation and inhibition of the $\alpha1^{N102Q}$ GlyR. A. Examples of currents activated by the indicated glycine concentrations in a cell expressing $\alpha1^{N102Q}$ GlyRs. B. Averaged potentiating dose-responses from 5 cells. The vertical axis is plotted as $(I - I_{EC30})/I_{max}$, where I_{EC30} is the control current in absence of tropisetron, I is the current in the presence of a given concentration of tropisetron and I_{max} is the maximum amplitude of the tropisetron-potentiated current. Parameters of best fit are given in the text. The corresponding curve for the α1 GlyR (dashed line) is included for comparison.

Figure 6. Determinants of tropisetron inhibitory sensitivity. A. Examples of the effect of indicated tropisetron concentrations on currents activated by EC_{30} glycine in cells expressing the indicated GlyRs. B. Averaged tropisetron inhibitory dose-response curves for the indicated GlyRs.

I_{max} is the maximum amplitude of the tropisetron-potentiated current. Parameters of best fit are given in Table 1. The corresponding curve for the α1 GlyR (dashed line) is included for comparison.

Figure 7. Structure-activity relationships of tropisetron analogues. A. Structures of tropisetron and related compounds investigated in this study. The granisetron structure is included as its binding site at the 5-HT_3R has been thoroughly investigated. B. Examples of the effect of indicated concentrations of atropine and SB203186 on currents activated by EC_{30} glycine in cells expressing α1 GlyRs. Note the complete absence of inhibition induced by SB203186. B. Averaged inhibitory dose-response curves for atropine at the α1 and $\alpha1^{N102Q}$ GlyRs. Parameters of best fit for the α1 GlyR curve are given in the text.

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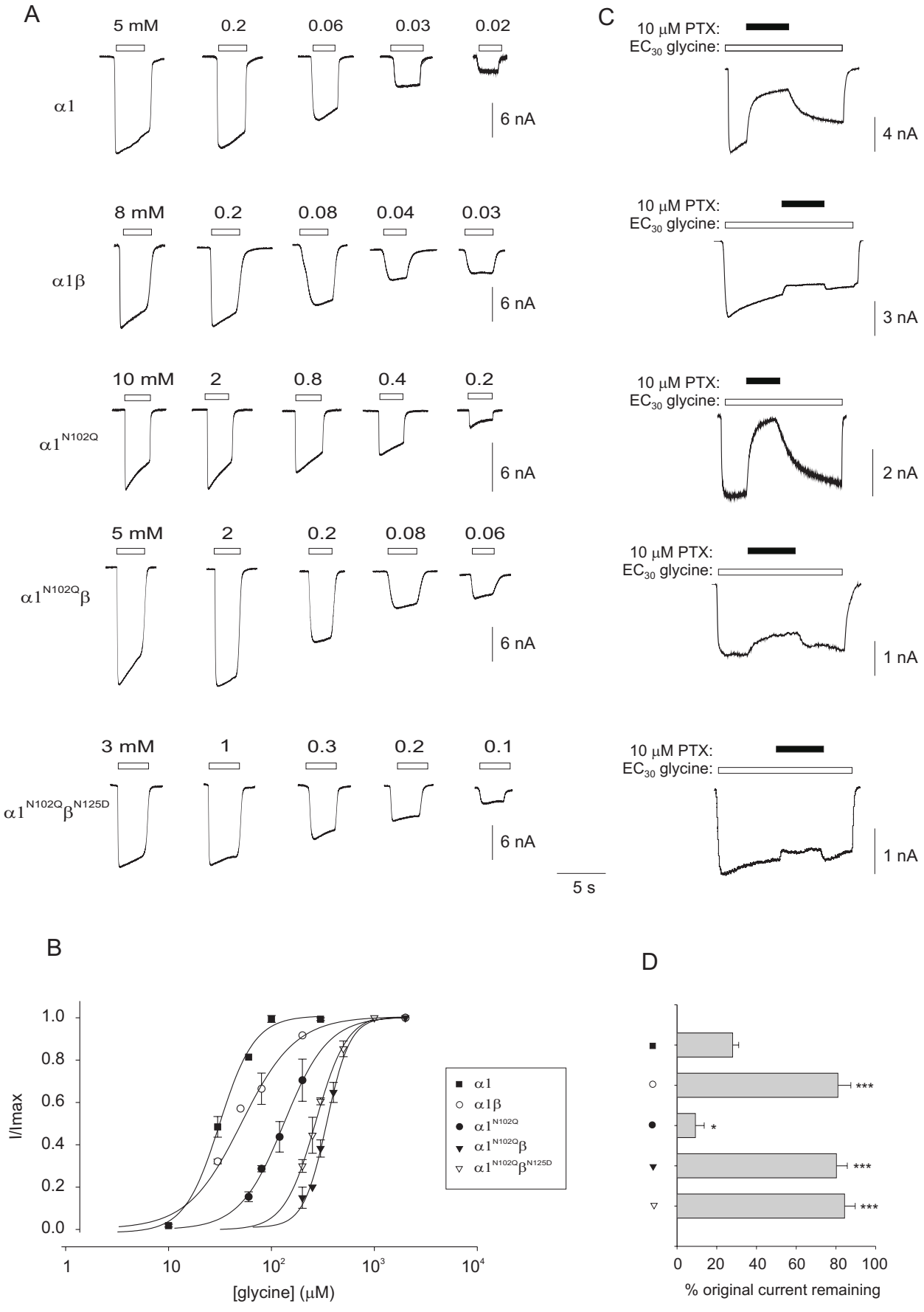
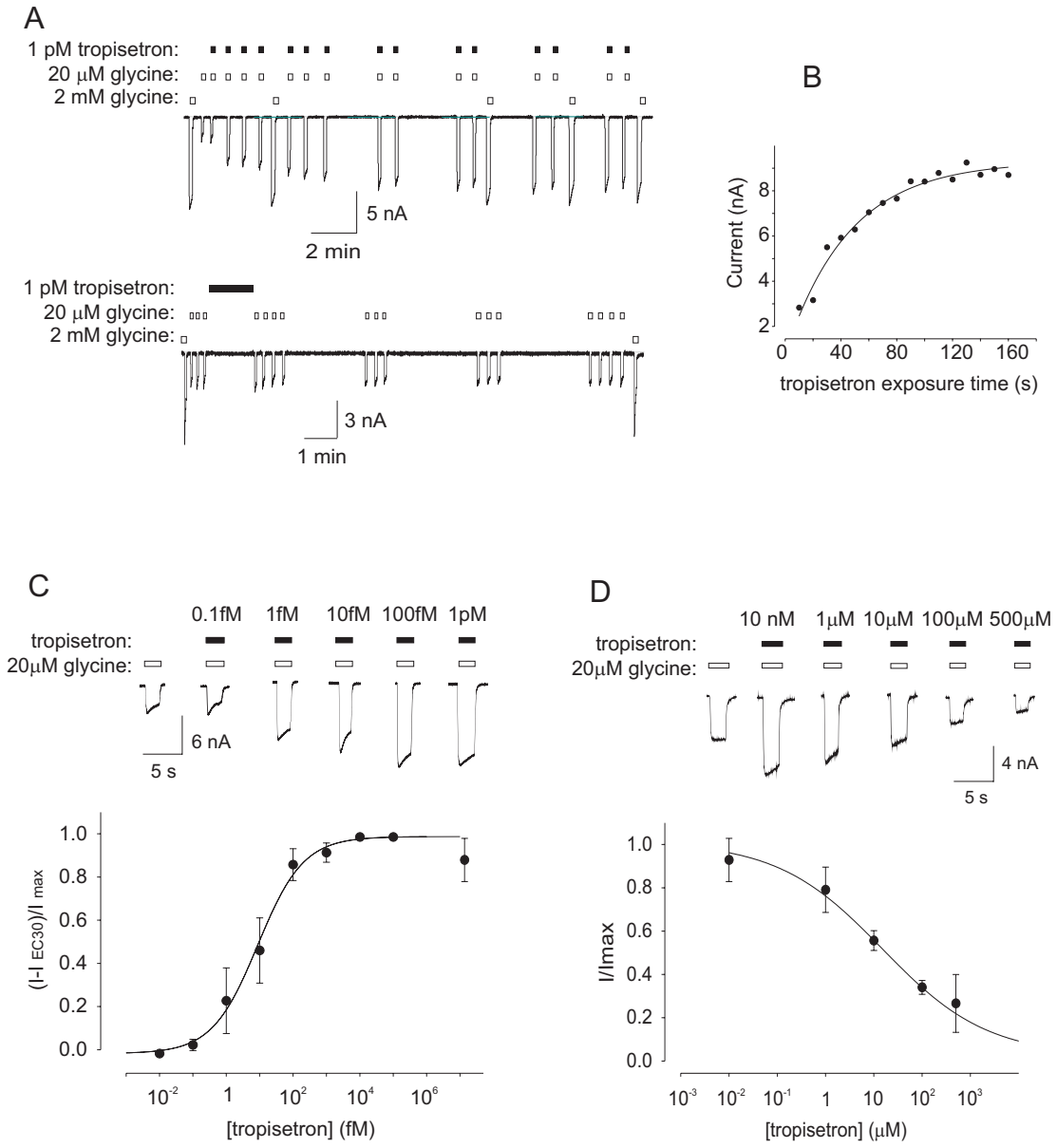


Figure 1



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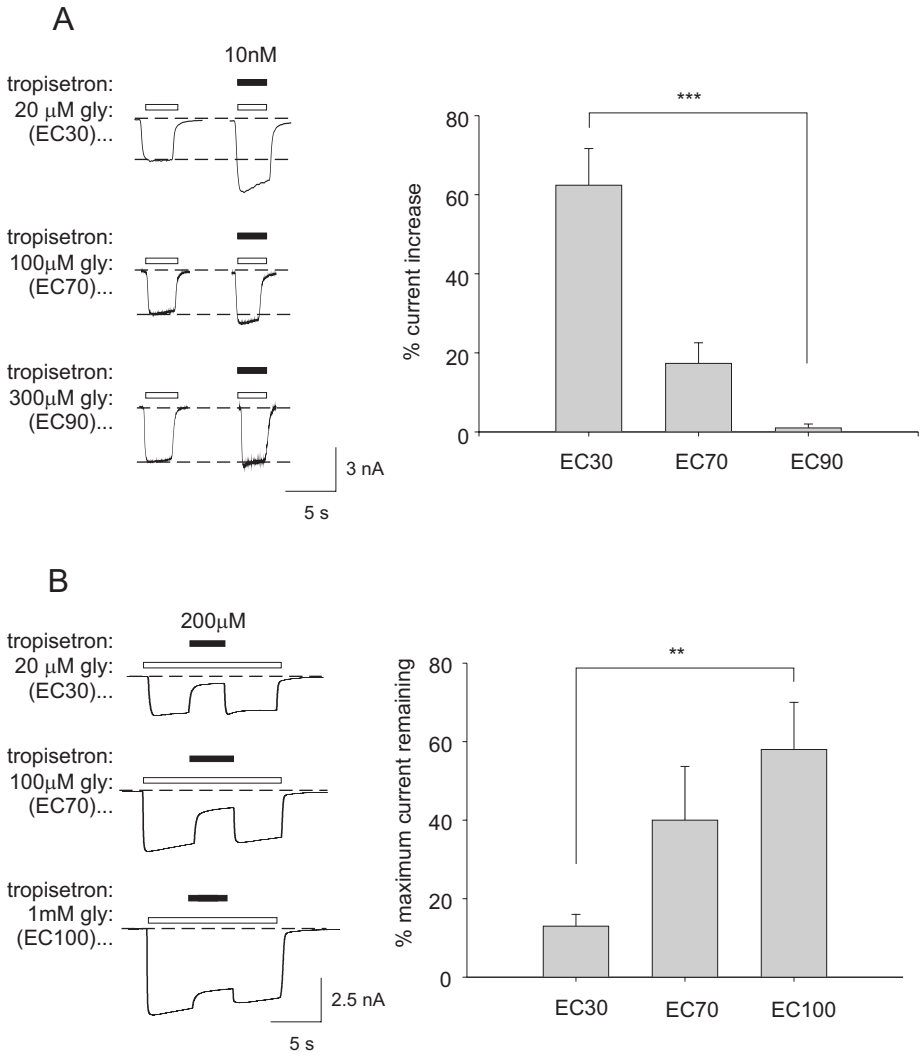

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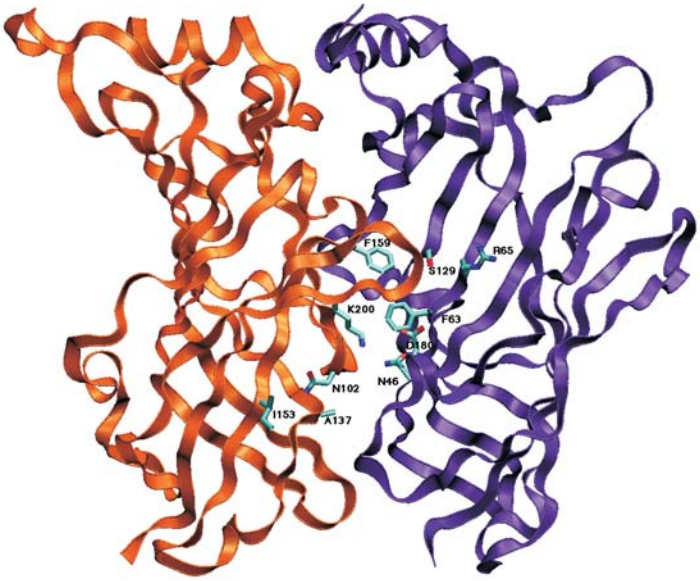

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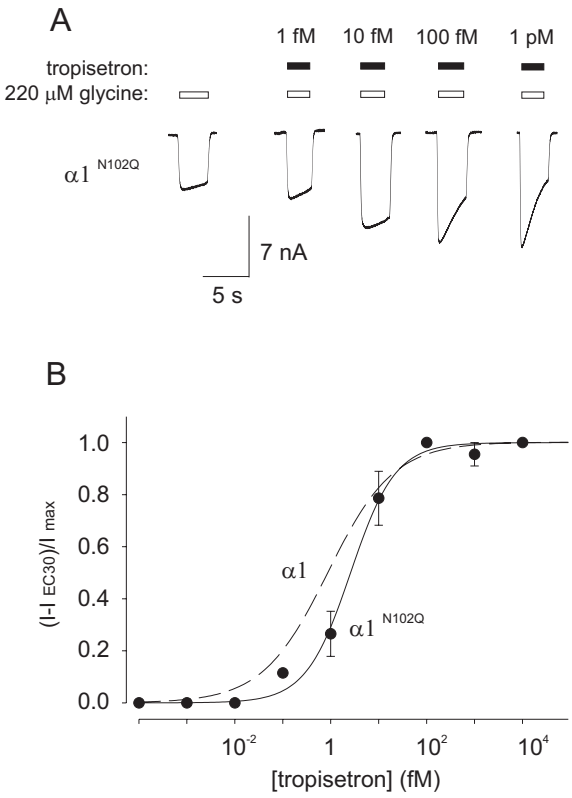

Figure 5

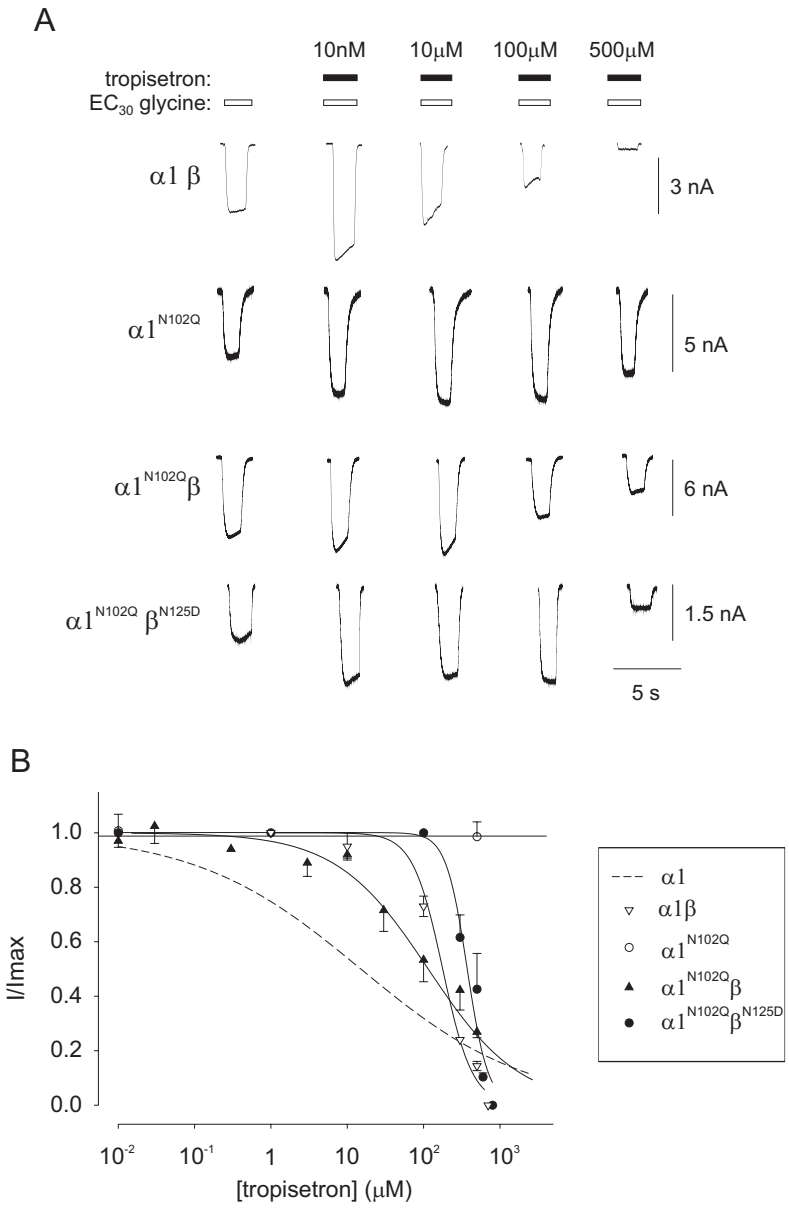

Figure 6

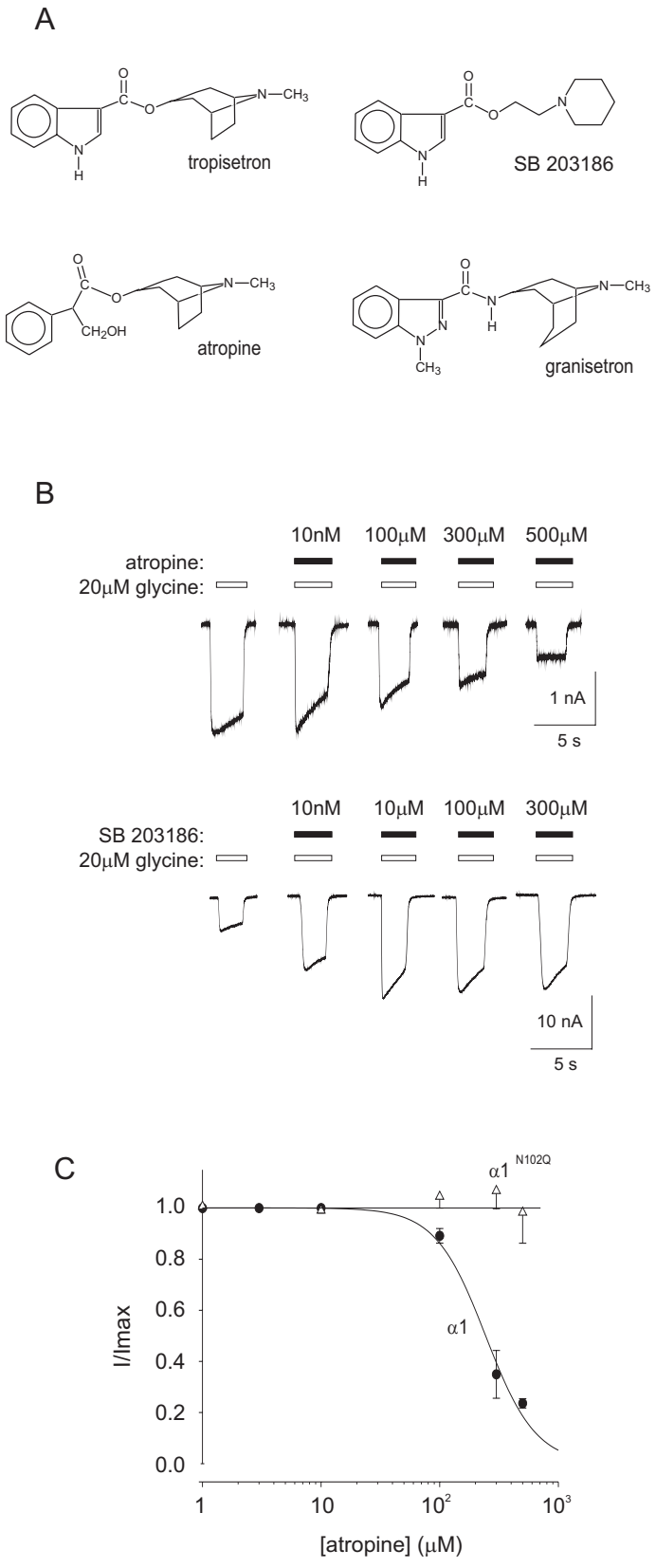

Figure 7