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The anticonvulsant stiripentol acts directly on the GABAA receptor as a positive allosteric modulator

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SUMMARY

Stiripentol(STP) has been used as co-therapy for treatment of epilepsy for many years. Its mechanism of action has long been considered to be indirect, as it inhibits the enzymes responsible for metabolism of other anticonvulsant agents. However, a recent report suggested that STP might also act at the neuronal level, increasing inhibitory GABAergic neurotransmission. We examined the effect of STP on the functional properties of recombinant $GABA_A$ receptors (GABARs) and found that it was a positive allosteric modulator of these ion channels. Its activity showed some dependence on subunit composition, with greater potentiation of α3-containing receptors and reduced potentiation when the β1 or ε subunits were present. STP caused a leftward shift in the GABA concentration-response relationship, but did not increase the peak response of the receptors to a maximal GABA concentration. Although STP shares some functional characteristics with the neurosteroids, its activity was not inhibited by a neurosteroid site antagonist and was unaffected by a mutation in the α3 subunit that reduced positive modulation by neurosteroids. The differential effect of STP on β1- and β2/β3-containing receptors was not altered by mutations within the second transmembrane domain that affect modulation by loreclezole. These findings suggest that STP acts as a direct allosteric modulator of the GABAR at a site distinct from many commonly used anti-convulsant, sedative and anxiolytic drugs. Its higher activity at α3-containing receptors as well as its activity at δ-containing receptors may provide a unique opportunity to target selected populations of GABARs.

Keywords

Anti-convulsant; GABA; electrophysiology; site-directed mutagenesis; recombinant; patch-clamp

INTRODUCTION

The anti-convulsant stiripentol (STP) is a novel anti-epileptic chemically unrelated to other drugs used to treat seizure disorders (Figure 1) (Trojnar et al., 2005;Chiron, 2007). It has been approved as an add-on therapy for the treatment of severe myoclonic epilepsy in infancy (Dravet syndrome) by the European Medicines Agency and can be effective in reduction of pharmacoresistant seizures. STP is an inhibitor of the hepatic cytochrome P450 enzymes involved in the metabolism of a variety of chemicals and its anti-convulsant actions were generally believed to arise from its ability to increase the effective concentrations of other, centrally-acting drugs and reduce toxic side-effects from their metabolites. However, STP does

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have anticonvulsant activity when administered alone, and some evidence suggests that STP may also have a direct neuronal mechanism of action. An earlier report indicated that STP might alter the activity of GABA transporters (Poisson et al., 1984), and a recent study in cultured hippocampal neurons demonstrated direct modulation of GABA-mediated post-synaptic currents by STP (Quilichini et al., 2006). These investigators showed that STP at clinically relevant concentrations caused slower decay of GABAergic IPSCs and an increase in the duration of channel openings.

The $GABA_A$ receptor (GABAR) is a ligand-gated chloride channel and is responsible for most of the fast inhibitory neurotransmission in the mammalian brain. The GABAR is the target for many drugs used clinically as anti-convulsants, sedatives, and anxiolytics. Therefore, direct positive modulation of these receptors by STP could underlie its anti-convulsant effects. The GABAR is notable for its substantial structural heterogeneity. Mammalian GABARs can contain subunits from seven different families with sixteen different subunit subtypes and the subunit composition of the receptor largely determines its pharmacological and functional properties (Korpi et al., 2002). Expression levels of the subunits and subtypes are differently regulated throughout the brain and change in response to physiological processes, such as development and learning, as well as pathological processes, such as epilepsy (Laurie et al., 1992; Wisden et al., 1992; Sperk et al., 2004). Drugs targeting defined receptor populations may therefore be extremely useful for selectively modulating neuronal activity in discrete brain regions without the general side-effects associated with less-selective GABAR modulators.

Most neurons express a wide number of GABAR subunits subtypes and therefore produce a heterogeneous population of receptors. The goal of our work was to determine whether the activity of STP is influenced by the subunit composition of the receptor and to further examine its mechanism of action at these receptors. We tested the effect of stiripentol on recombinant GABARs containing a variety of different subunit combinations with patch-clamp recordings from transiently transfected mammalian cells.

METHODS

Transfection of HEK-293T cells

Full-length cDNAs for the rat GABAR α1, α3–α6, β1–beta;3, γ1–γ3, δand human α2 and ε subunits in pCMV, pCDNA1.1Amp, or pCDM8 expression vectors were transfected into the human HEK-293T cell line. For selection of transfected cells, the plasmid pHook™-1 (Invitrogen, San Diego, CA) was also transfected into the cells. HEK-T cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were passaged by a 2 min. incubation with 0.25% trypsin/0.1% EDTA solution in phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH = 7.3).

The cells were transfected using calcium phosphate precipitation. Plasmids encodingGABAR subunit cDNAs were added to the cells in 1:1:1 ratios of 2 μg each plus 1 μg of the plasmid encoding sFv. Following a 4–6 hr. incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 sec. The selection procedure for pHook expression was performed 44–52 hrs. The cells were passaged and mixed with 3–5 μl of magnetic beads coated with antigen for the pHook antibody (approximately 6×10^5 beads) (Chesnut et al., 1996). Following a 30–60 min. incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into DMEM, plated onto glass coverslips treated with poly L-lysine and coated with collagen and used for recordings 18–28 hrs. later.

Electrophysiological recording solutions and techniques

For whole-cell recording the external solution consisted of (in mM); 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. Recording electrodes were filled with an internal solution of (in mM); 153 KCl, 1 $MgCl_2$, 5 K-EGTA (ethylene glycol-bis (β-aminoethyl ether N,N,N′N′-tetraacetate) and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. These solutions provided a calculated chloride equilibrium potential of −1.4 mV. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota FL) on a two-stage puller (Narishige, Japan) to a resistance of 5–10 MΩ. Drugs were applied to cells using a stepper solution exchanger with a complete exchange time of 20–30 msec (SF-77B, Warner Instruments, Hamden CT). There was continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B (Foster City, CA) patch clamp amplifier and stored on a computer hard drive for off-line analysis. All experiments were performed at room temperature (near 25° C).

Drugs were diluted from frozen stocks in water (GABA) or made fresh in DMSO (STP, 17-phenyl-(3α,5α)-androst-16-en-3-ol (17-PA) and alphaxalone) on the day of the experiment. DMSO at the highest concentration used (1%) had no impact on the activity of α3β3γ2L receptors (101.7 ± 1.8% of the response to 10 μM GABA alone, N=9). Most experiments were performed using DMSO at a concentration of 0.01% or less.

Analysis of whole-cell currents

Whole-cell currents were analyzed off-line using the programs Clampfit (pClamp8 suite, Axon Instruments, Foster City CA) and Prism (Graphpad, San Diego, CA). Normalized concentration-response data for responses to GABA or STP were fit with a four-parameter logistic equation (Current = (Minimum current + (Maximum current-Minimum current))/(1 +(10^(log EC_{50}− log [drug])*n) where n represents the Hill number. All fits were made to normalized data with the current expressed as a percentage of the maximum current elicited by saturating GABA concentrations for each cell or, in the case of modulation by stiripentol, to the response to GABA alone. Statistical tests were performed using the Instat program (Graphpad). Differences among isoforms were determined with the Tukey-Kramer multiple comparisons test with a minimum p value for significance of 0.05.

Generation of mutations

Complimentary oligonucleotide primers encoding the mutation sites were synthesized by the University of South Carolina DNA synthesis core facility (Columbia, SC) or by Integrated DNA Technologies (Coralville, IA). Point mutations were generated with the commercially available QuikChange kit (Stratagene, La Jolla, CA) and were verified by sequencing (Univ. of South Carolina sequencing core facility).

RESULTS

Stiripentol potentiates the response of recombinant receptors to GABA

To determine if stiripentol(STP) could directly modulate the activity of recombinant $GABA_A$ receptors (GABARs), STP was co-applied with GABA to transiently transfected HEK-293T cells. STP concentrations from 0.1 μM – 1 mM were tested on cells expressing either the α1β3γ2L or α3β3γ2L receptor isoforms. These subunit combinations were selected for complete STP concentration-response relationships because the α1β3γ2L isoform represents one of the most commonly occurring native subunit combinations (McKernan et al., 1996) and our studies on subunit subtype dependence showed that STP was most effective at α3-containing receptors compared to those with other α subtypes (see Figure 3).

At both receptor isoforms, STP caused substantial potentiation of the response to GABA alone (Figure 2). The average EC_{50} for potentiation of the α3β3γ2L receptors was 24.6 ± 7.0 μM (N=5) while that for α1β3γ2L was 35.5 ± 4.9 μM (N=3). The EC_{50} values were determined by fits to the data excluding the points at 1 mM STP. These concentrations correspond to about 6–9 mg/L, which is well within the range of STP plasma and brain levels at which therapeutic effects are observed (Shen et al., 1992;Trojnar et al., 2005). The maximum amount of potentiation was significantly greater at α3-containing receptors but the peak response was obtained at 100 μM STP for both receptor isoforms (Figure 2B). At STP concentrations higher than 100 μM a reduced potentiation was commonly observed and the currents often showed enhanced desensitization and a rebound current following removal of the drugs (Figure 2A). This is consistent with an open channel block at these higher concentrations, a characteristic commonly observed with many GABAR modulators (Wofford et al., 1994; Thompson et al., 1996;Hill-Venning et al., 1997). Since 100 μM STP was found to be the most effective concentration at both these isoforms this concentration was used for all subsequent studies of its potentiating effects.

The amount of potentiation is influenced by the subunit composition of the receptor

The subunit composition of the GABAR is a major determinant of both its functional and its pharmacological properties and the activity of nearly all GABAR modulators is influenced by the subunit family and/or subtype (Korpi et al., 2002). To characterize the subunit subtype-dependence of STP, we compared its effect on thirteen receptor isoforms containing different combinations of GABAR subunits.

α subunit subtype—The α subunit family of the GABAR has six different subtypes, making it the most diverse of the families. Each of these six subtypes was co-expressed with β3 and γ2L subunits to produce recombinant receptors that differed only in their α subtype. 100 αM STP was co-applied with a concentration of GABA that produced a sub-maximal response (~$EC_{10–20}$) for each isoform. All six isoforms showed robust potentiation by STP (Figure 3). The α3-containing receptors exhibited the greatest potentiation, with a response significantly greater that that observed with any other isoform (Figure 3B). All other α subtypes showed responses similar to one another. Although STP does not appear to have a strict dependence on α subtype, in that all isoforms showed some responsiveness, it does preferentially potentiate receptors containing the α3 subunit. Because of this, all subsequent studies were performed with α3-containing receptors.

β subunit subtype—The three different β subtypes were co-expressed with α3 and γ2L subunits to determine their effect on STP activity. All combinations were potentiated by 100 μM STP, but STP was significantly less effective at receptors containing the β1 subtype (Figure 4). There was no difference between the responses of receptors containing β2 or β3 subtypes. This pattern of selectivity is not uncommon. A variety of GABAR modulators, including loreclezole, etomidate, etifoxine and many anti-inflammatory drugs, are also less effective at β1-containing receptors (Wafford et al., 1994;Belelli et al,. 1997;Hill-Venning et al., 1997;Hamon et al., 2003: Smith et al., 2004).

γ subunit subtypes and αβ heteromers—We also examined the role of the tertiary subunit on responsiveness to STP. The identity of the α subtype did not alter the response of the receptors, as all three isoforms showed a similar level of potentiation by 100 μM STP (Figure 5). Receptors containing the γ3 subunit did appear to be more affected by the inhibitory action of STP, with a prominent rebound current when the drugs were removed (Figure 5A). The presence of a γ subunit was not necessary for responsiveness to STP, as both α3β3 and α3β3γ receptors were potentiated to a similar degree (Figure 5A, 5B).

δ and ε subunits—The δ and ε subunits are associated with relatively small populations of neuronal receptors, but confer distinct characteristics that might make them important therapeutic targets. The δ subunit is associated with extrasynaptic receptors that produce tonic current, especially in the thalamus and the granule cells of the hippocampus and cerebellum (Glykys et al., 2007). The α3β3δ receptor isoform was highly potentiated by 100 μM STP and showed the largest percentage potentiation of all the α3-containing isoforms (Figure 5A, 5C). Receptors containing the ε subunit are uniquely characterized by a substantial amount of spontaneous, agonist-independent, channel activity (Neelands et al., 1999). This spontaneous current can be inhibited by open channel blockers such as picrotoxin or zinc. We examined the effect of 100 μM STP on both the spontaneous and the GABA-evoked channel activity. The spontaneous activity was measured by the reduction in holding current produced by either 100 μM picrotoxin or 1 mM zinc. The average spontaneous current was 15.0 ± 4.1% of the maximum evoked response, measured as the response to 1 mM GABA (N=7). Both zinc and picrotoxin produced similar reductions in holding current. The ε-containing receptors were relatively insensitive to potentiation by STP, with a similar enhancement of the spontaneous (agonist-independent) and GABA-evoked currents (Fig 5A, 5C). The ε subunit confers resistance to many GABAR modulators (Ranna et al., 2006), a property that may be due to its high spontaneous activity rather than a lack of binding sites or transduction pathways.

Stiripentol shifts the GABA concentration response curve to the left but does not increase the maximal response

Some GABAR modulators, such as the barbiturates, can increase the maximum response of the channel to saturating GABA concentrations. Others, like the benzodiazepines, increase the affinity of the receptor for GABA, but do not increase the maximum response. We examined the effect of 100 μM STP on the response of α3β3γ2L receptors to GABA across a concentration range from 0.1 μM to 3 mM. STP produced greater potentiation at low concentrations of GABA and did not increase the peak response to maximum GABA levels (Figure 6). The concentration-response relationship to GABA was shifted to the left by STP, with an average GABA EC_{50} of 49.0 ±8.4 μM (N=4) with GABA alone and 6.7 ± 1.9 μM (N=5) when GABA was co-applied with STP ($p < 0.01$, unpaired t-test).

Effect of membrane voltage on potentiation by stiripentol

The activity of some allosteric modulators is influenced by membrane voltage. We compared the potentiation of the response to 10 μM GABA by 100 μM STP at −50 and +50 mV (Figure 7). We did find a substantial reduction in the amount of potentiation at positive potentials, although the response was still significantly greater than with GABA alone. The reduced potentiation was most likely due a voltage-dependent leftward shift in GABA sensitivity of the receptors (Fisher, 2002). Most recombinant GABARs exhibit outward rectification in their responses to a sub-maximal concentration of GABA, but no rectification in the peak response. Since the amount of potentiation by STP depends upon the effective concentration (EC) of GABA co-applied (Figure 6), less potentiation would be expected at positive than at negative membrane potentials when using the same concentration of GABA, as it represents a higher EC at positive potentials. Therefore, it does not appear that STP action is directly influenced by membrane voltage.

Stiripentol can directly activate the $GABA_A$ receptor

Many positive allosteric modulators of $GABA_A$ receptors can also act as agonists at high concentrations. To determine if STP could directly activate the receptor, 1 mM STP was applied to cells expressing the α3β3γ2L isoform (Figure 8). All cells (N= 13) responded to STP with a current averaging 12.4 ± 2.0% of the response to 3 mM GABA. STP thus appears to act as a weak partial agonist at these receptors, and its relative efficacy at the α3-containing receptors

is comparable to that of other GABAR modulators with agonist activity, including many intravenous and inhaled anaesthetics and neurosteroids (Hill-Venning et al., 1997;Schofield et al, 2005;Drafts et al., 2006;Hosie et al., 2006).

Does stiripentol act though GABAR sites used by other positive allosteric modulators?

The subunit-subtype dependence exhibited by STP shares some characteristics with other GABAR modulators. Several different types of drugs have a greater activity at β2/β3-containing receptors compared to β1-containing receptors (see Smith et al., 2004). In some cases, this difference is conferred by a variable serine/asparagine residue within the second transmembrane domain of the β subunits. Mutation of this residue in the β subunits has been shown to exchange sensitivity to loreclezole (Wingrove et al., 1994), etomidate (Belelli et al., 1997), and several anaesthetics (Cestari et al., 2000). We co-expressed mutated $\beta1_{(S265N)}$ and $\beta3_{(N265S)}$ subunits with α3 and γ2L to determine if this site was responsible for the reduced STP sensitivity associated with the β1 subunit. Neither mutation affected the ability of 100 μM STP to potentiate the response to GABA (Figure 9).

Because of the greater activity of STP at receptors containing the α3 subunit, we also examined the potential role of a transmembrane residue unique to the α3 subtype. The amino acid sequences of the transmembrane domains are highly conserved among the α subtypes, and only one residue within in these structures, a methionine in the third transmembrane domain, is unique to the α3. This methionine is present in the α3 subunit because of RNA editing, as the DNA sequence encodes the isoleucine found in all other subtypes (Ohlson et al., 2007). The editing is developmentally regulated, so that nearly all α3 subunits in the adult brain incorporate a methionine residue. The functional significance of this structural variation is unknown. We found that the activity of STP was unaffected by the presence of an isoleucine residue at this location (Figure 9).

The mechanism of action and subunit dependence of STP activity is similar to that of many neurosteroids that act as positive allosteric modulators. To determine if STP shares a binding site and/or structural requirements with the neurosteroids, we examined the effects of a competitive antagonist and a mutation within the α3 subunit that reduce potentiation by neurosteroids. The competitive antagonist 17-PA (17-phenyl-(3α,5α)-androst-16-en-3-ol) selectively inhibits potentiation of GABARs by 5α-reduced steroids (Mennerick et al., 2004). 10 μM 17-PA was co-applied with GABA or GABA + 100 μM STP to cells expressing the α3β3γ2L receptor isoform (not shown). 17-PA had no effect on the potentiation by STP, with the current amplitude averaging 102.0 ± 4.0% (N=4) of the response to GABA + STP in the absence of 17-PA. We found that this concentration of 17-PA blocked about 50% of the potentiation by 1 μM alphaxalone at these receptors and had no effect on the response to GABA alone, consistent with the initial report (Mennerick et al., 2004). In addition, several studies have identified structures within the GABAR subunits that influence the activity of neurosteroids (Hosie et al., 2007). Within the α subunit, mutation of a conserved glutamine residue in the first transmembrane domain to tryptophan eliminates sensitivity of the receptor to potentiation by neurosteroids but does not affect the response to benzodiazepines or barbiturates (Hosie et al., 2006). We found that this mutation within the α3 subunit did not affect the ability of STP to potentiate the receptor (Figure 9), but did prevent potentiation by 1 μM alphaxalone (N=2, not shown). These findings are consistent with the conclusion of Quilichini et al. (2006) that STP is unlikely to act through the same site as the neurosteroids.

Can stiripentol enhance the response of GABARs to benzodiazepines?

Stiripentol is currently approved only as an add-on therapy to improve seizure control when a single pharmacotherapy is unsuccessful (Chiron, 2007). Very commonly, it is co-administered with benzodiazepine anti-convulsants. Because of STP's action on metabolic enzymes, it was

presumed to enhance the effectiveness of these drugs by increasing plasma concentrations and reducing the levels of toxic by-products. However, since both drugs act through the GABAR, we examined whether co-administration of STP with diazepam would further enhance the potentiation of the GABAR current. Maximally effective concentrations of STP (100 μM) and diazepam (1 μM) were applied either separately or together to cells expressing α3β3γ2L (Figure 10). The current amplitude was increased even further when both were applied, suggesting that they act through separate mechanisms which can effectively add together to increase channel activity.

DISCUSSION

These data show that stiripentol is a positive allosteric modulator acting directly upon the $GABA_A$ receptor. STP acts to increase the sensitivity of the receptor to GABA, without increasing the maximum response to saturating GABA concentrations. STP can also directly activate the receptor, as a weak partial agonist. The activity of STP showed some dependence upon the subunit composition of the receptor, with α3 or δ-containing receptors showing the greatest response, but was able to potentiate receptors containing any of the α, β, or γ subtypes and did not require the presence of a γ subunit.

STP appears to act through a unique site which is clearly distinct from many widely used GABAR modulators. Quilichini et al. (2006) suggested that STP might act on GABARs through a barbiturate-like mechanism. While there are some similarities, including shared effects on channel open duration, the ability to act as an agonist, and the appearance of channel block at high concentrations, there are also some key differences. STP has a pattern of subunit-subtype dependence distinct from that of the barbiturates (Thompson et al., 1996) and does not increase the maximum response of the receptors. We have shown that mutations at sites in the α and β subunits that affect the activity of loreclezole, neurosteroids and many anaesthetics do not impact modulation by STP. STP may share characteristics with some of the other α3-preferring drugs that have been developed (Meadows et al., 1998; Dias et al., 2005), a question that can be better addressed when structural sites or antagonists are identified that are selective for these agents. With our current understanding of the structural requirements for subtype-selective drugs, it is very difficult to predict which compounds might show selective action. Further information regarding the binding sites or transduction pathways will therefore benefit the rational design of new, selective drugs.

STP does not show a strict dependence on the subunit composition of the receptor, as all subunit combinations tested were potentiated to some extent. However, STP was most effective at receptors containing the α3 or δ subunits and these isoforms might therefore be selectively targeted with lower concentrations of STP. Since some studies suggest that the α3 subunit is not necessary for the anti-convulsant action of benzodiazepines (Möhler et al., 2002), drugs acting at these receptor populations may be more useful for other therapies, including anxiety disorders and neuropathic pain. The α3 subunit is highly expressed in embryonic brain, but has a restricted expression in the adult, where it is found primarily in the cortex (Laurie et al., 1992; Wisden et al., 1992). This subunit has received attention for its potential role in a number of neurological disorders. Changes in α3 expression are observed during epileptogenesis and following seizure onset in several forms of epilepsy (Brooks-Kayal et al,. 1998; Poulter et al., 1999; Loup et al., 2006) and animals lacking the α3 subunit exhibit deficits similar to those observed in schizophrenic patients (Yee et al., 2005). Because of its limited expression pattern in the adult brain, it has been suggested that drugs targeting the α3-containing receptors may provide selective anxiolysis with fewer side-effects (see Dias et al, 2005). In addition, very recent studies suggest a potentially important role for α3-containing GABARs in spinal cord in the reduction of neuropathic and inflammatory pain (Knabl et al., 2008).

The δ subunit primarily co-assembles with the α4 and α6 subunits to produce high affinity receptors with slow desensitization kinetics (Glykys et al, 2007). The tonic current generated by these receptors in response to ambient levels of GABA can produce a powerful inhibitory drive in the hippocampus, cerebellum and thalamus. Changes in expression of the δ subunit are observed in several seizure disorders with concomitant changes in the level of tonic current or its sensitivity to δ-selective modulators (Peng et al., 2004; Qi et al., 2006; Zhang et al., 2007). These receptors also have unique pharmacological properties. Although they are insensitive to benzodiazepines, they may represent a primary site of action for the behavioral effects of neurosteroids, ethanol and many anaesthetics (Glykys et al., 2007). Since this population of receptors is sensitive to modulation by STP, co-therapy of STP with the benzodiazepines may provide substantive additive benefits by allowing modulation of both synaptic and extra-synaptic receptors. Our results also show that STP can further enhance the action of benzodiazepines within the same receptor population, suggesting an additional mechanism for its anti-convulsant effects.

Effective treatment of many disorders of neuronal hyperexcitability is often limited by side-effects of the drug therapy. Recent efforts in drug development have focused on finding more selective modulators of small populations of GABAR receptors (Korpi et al., 2006). These efforts might be particularly fruitful if those targets are particularly associated with damaged or dysfunctional tissue. Further work is needed to understand the roles of different GABAR subunits and subtypes in both normal and pathological conditions in order to provide rational and selective treatment of different neurological disorders.

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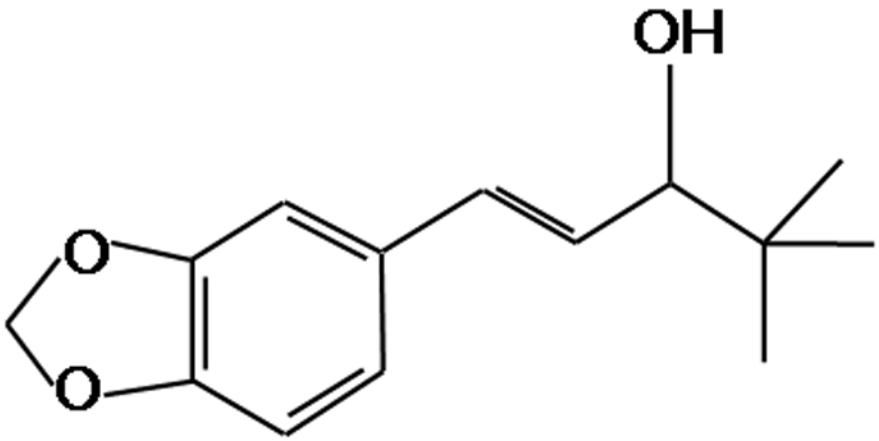

Figure 1. Structure of Stiripentol

STP is an aromatic allylic alcohol (4,4-dimethyl-1-[3,4(methylenedixy)-phenyl]-1-penten-3-ol) chemically unrelated to other clinically used anti-convulsants.

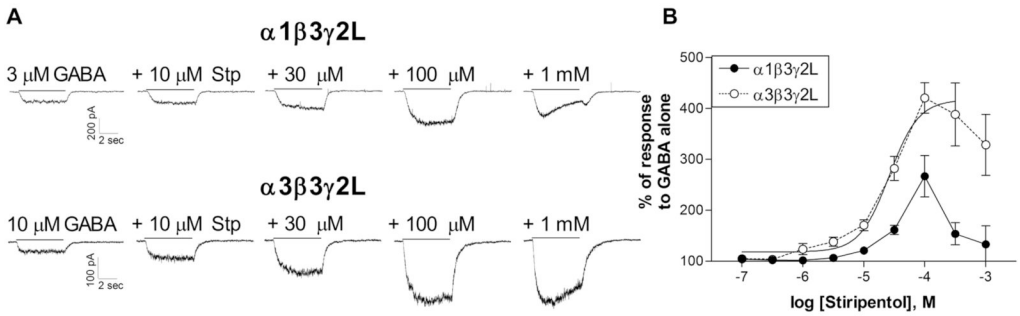

Figure 2. Stiripentol increases the response to GABA in a concentration dependent manner

HEK-293T cells were transfected with the α1 or α3 subunit along with β3 and γ2L subunits. The peak current response to 5 sec applications of GABA alone or with stiripentol (+ Stp) was measured in cells voltage-clamped at −50 mV. The GABA concentration used represented $EC_{10–20}$ for each isoform.

A. Representative traces in response to GABA and GABA with increasing concentrations of stiripentol. The amplitude of the current was increased by STP in a concentration-dependent manner. STP was co-applied with GABA and the onset of the potentiation was often delayed, requiring two to three consecutive applications to observe the peak effect at each concentration. However, the potentiation was readily reversible with a 1 min. wash. High concentrations of STP often produced less potentiation or inhibition, with a 'rebound' current following application. All traces for each α subtype shown were obtained from the same cell.

B. Concentration-response relationships were constructed by measuring the peak current with co-application of stiripentol as a percentage of the response to GABA alone for each cell. Symbols and bars represent the mean ± SEM. Excluding the response to 1 mM STP, the averaged data shown above was fit with a four-parameter logistic equation. The EC_{50} from this fit for the α3β3γ2L isoform was 26.1 μM (N=5), while the EC_{50} from the fit of the α1β3γ2L data was 31.4 μM (N=3, fit not shown).

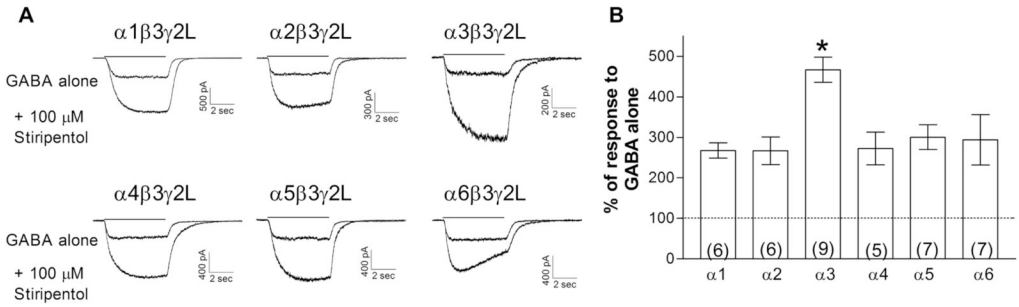

Figure 3. Effect of the α subtype on potentiation by stiripentol

A. Representative traces from receptors composed of β3, γ2L and one of the α subtypes, as indicated, showing the current response to GABA alone or GABA + 100 μM STP from transfected cells voltage-clamped at −50 mV. GABA concentration was 0.3 μM (α6), 1 μM (α4, α5), 3 μM (α1, α2) or 10 μM (α3), representing an $EC_{10–20}$ for each isoform (Picton and Fisher, 2006).

B. Bars represent the mean potentiation (± SEM) of receptors containing different α subtypes by 100 μM STP. The dashed line indicates the response to GABA alone. The number in parentheses gives the number of cells. * indicates a significant difference from all other isoforms ($p < 0.05$).

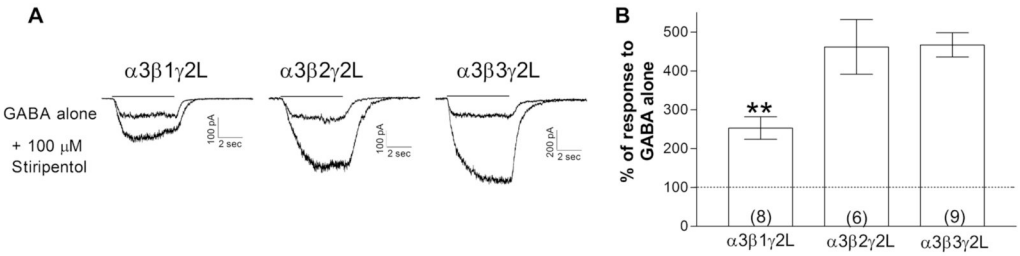

Figure 4. Effect of the β subtype on potentiation by stiripentol

A. Representative traces from receptors composed of α3, γ2L and one of the β subtypes, as indicated, showing the current response to GABA alone or GABA + 100 μM STP from transfected cells voltage-clamped at −50 mV. The traces shown for α3β3γ2L are the same as in Figure 3. GABA concentration was 30 μM (β1) or 10 μM (β2/β3), representing an $EC_{10–20}$ for each isoform.

B. Bars represent the mean potentiation (± SEM) of receptors containing different β subtypes by 100 μM STP. The dashed line indicates the response to GABA alone and the number in parentheses gives the number of cells. ** indicates a significant difference from the two other isoforms ($p < 0.01$).

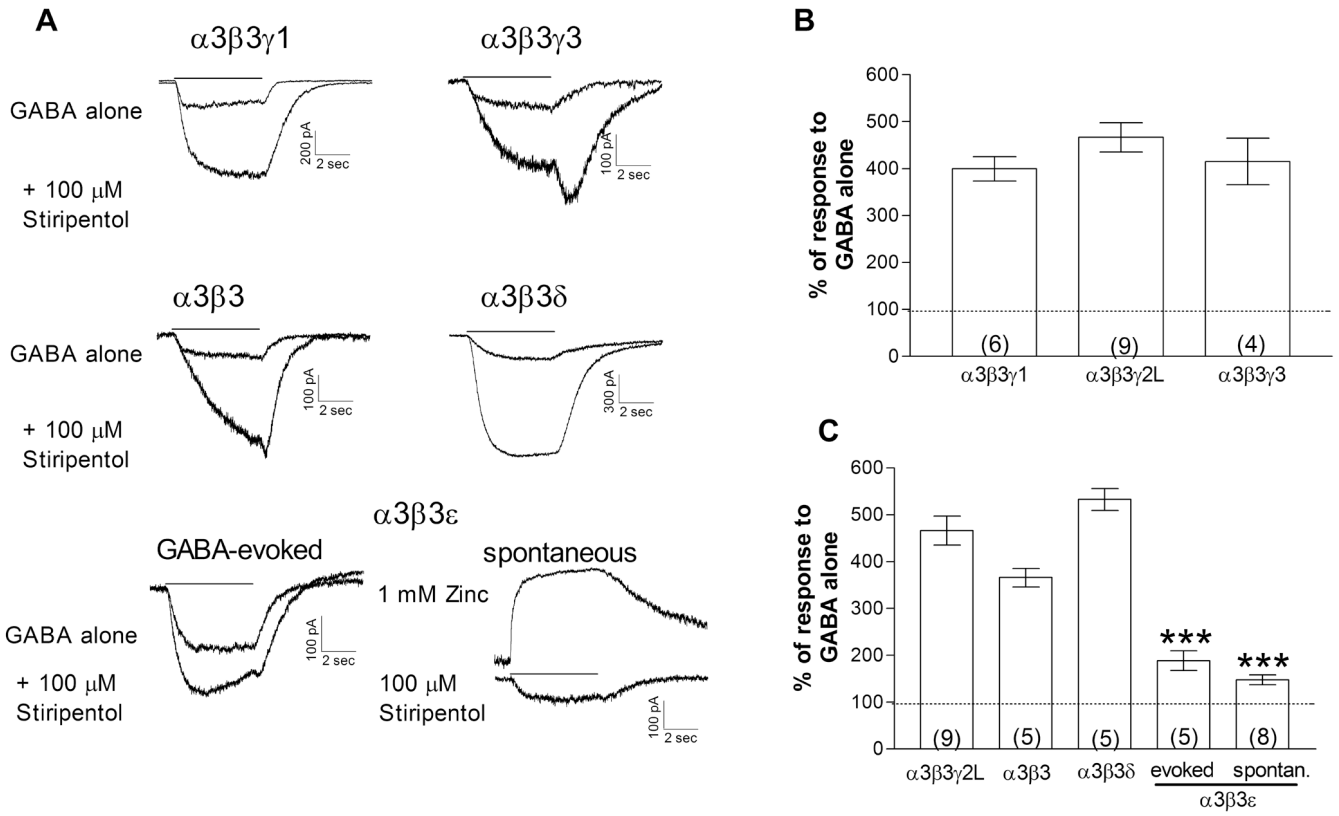

Figure 5. Effect of the γ, δ and ε subunits on stiripentol potentiation

A. Representative current traces are shown from receptors composed of α3 and β3 subunits, with or without a tertiary subunit as indicated. Potentiation of the GABA-evoked response was determined with 5 second applications of GABA alone or GABA + 100 μM STP. To measure the spontaneous activity of ε-containing receptors, 1 mM zinc or 100 μM picrotoxin was applied in the absence of GABA. The effect of STP on this agonist-independent activity was determined by application of 100 μM STP in the absence of GABA. Cells were voltage-clamped at −50 mV. GABA concentration was 0.03 μM (ε), 0.1 μM (αβ, δ) or 3 μM (γ1/γ3), representing an $EC_{10–20}$ for each isoform.

B. Bars represent the mean potentiation (± SEM) of receptors containing different subunit combinations by 100 μM STP. The dashed line indicates the response to GABA alone and the number in parentheses gives the number of cells. *** indicates a significant difference from all other isoforms ($p < 0.001$).

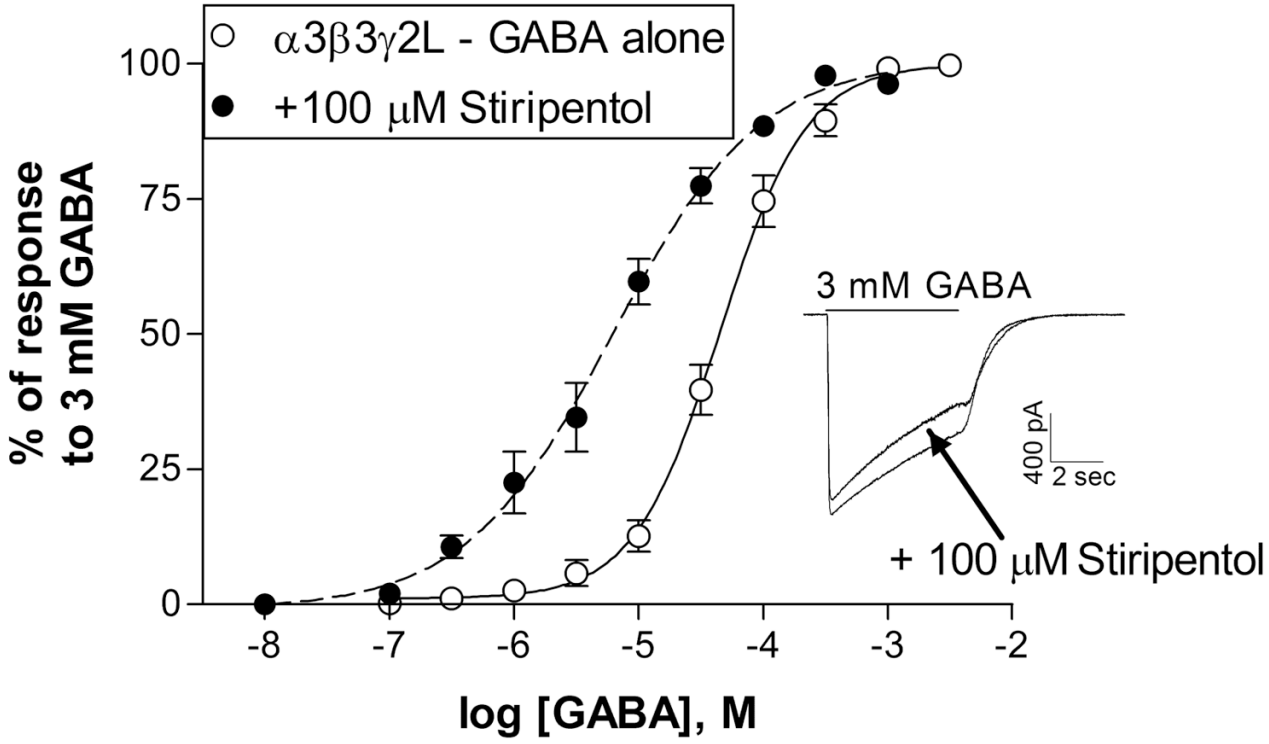

Figure 6. Stiripentol increases sensitivity to GABA but does not increase the maximum response

GABA concentration-response relationships were determined in the absence and presence of 100 μM STP. The response to each GABA concentration was normalized for each cell to the response to 3 mM GABA alone. STP caused a leftward shift in GABA sensitivity but did not increase the peak response. Inset traces show a representative current response to 3 mM GABA in the presence and absence of 100 μM STP. GABA EC_{50} values from the fits shown to averaged data are 45.2 μM with GABA alone (N = 4) and 6.2 μM in the presence of STP (N = 5).

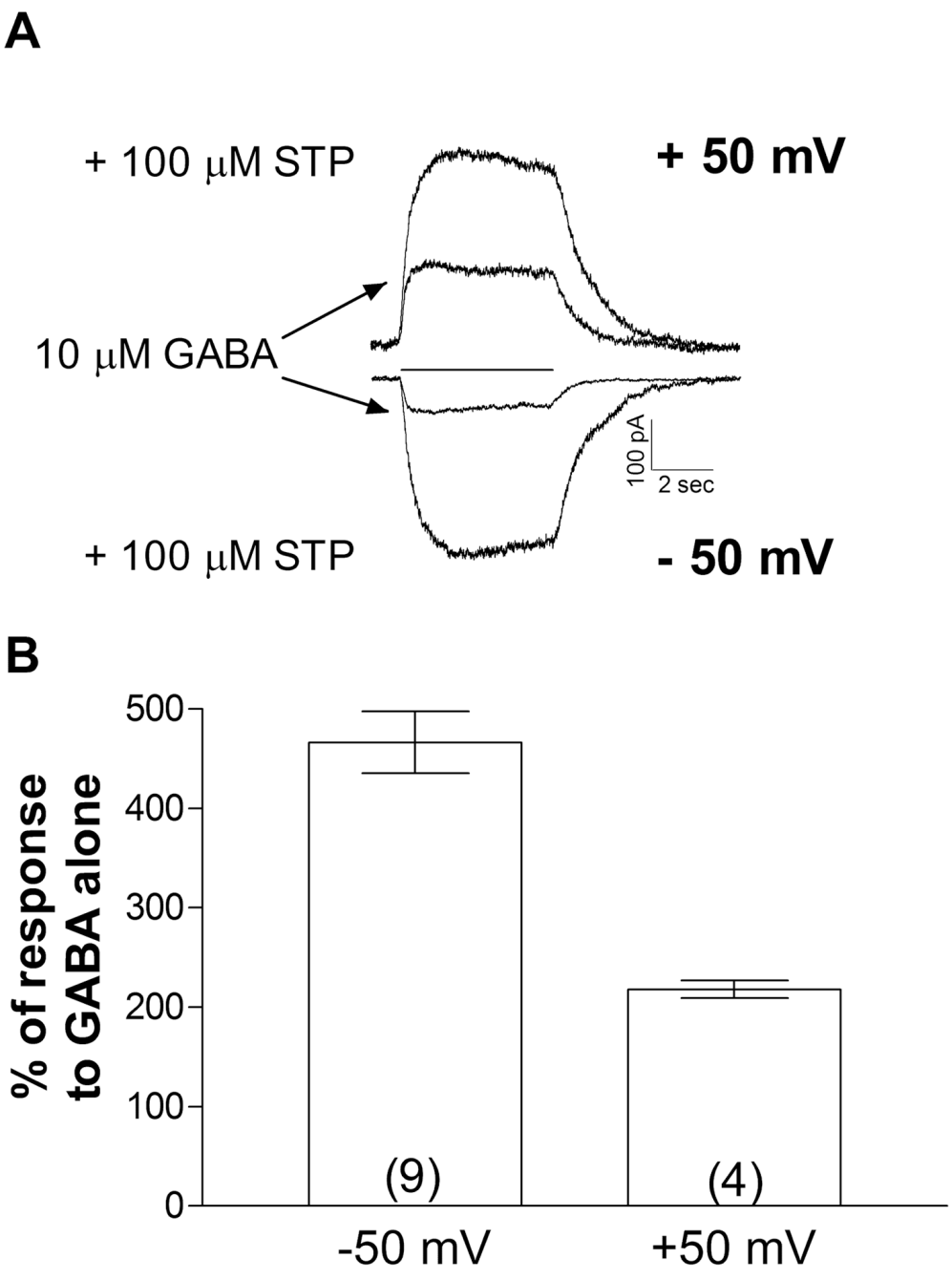

Figure 7. Effect of membrane voltage on STP potentiation of α3β3γ2L receptors

A. Currents are shown in response to 10 μM GABA or 10 μM GABA + 100 μM STP at a membrane potential of either −50 mV or + 50 mV. The reversal potential for the response was near 0 mV. Responses to sub-maximal concentrations of GABA exhibit outward rectification, with larger currents at positive membrane potentials. All responses shown are from the same cell.

B. Bars represent mean ± SEM for potentiation by 100 μM STP of the response to 10 μM GABA at either +50 or −50 mV. Data was not necessarily obtained from the same cells under each condition. Number of cells is indicated by the number in parentheses.

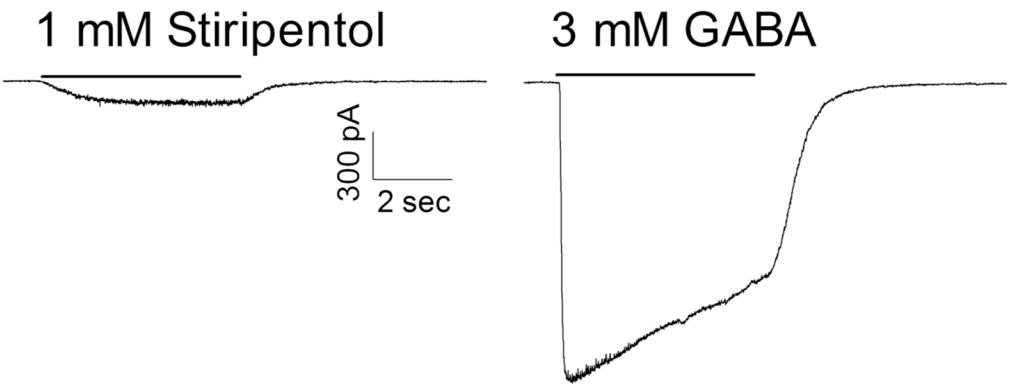

Figure 8. STP directly activates the $GABA_A$ receptor

Representative currents are shown in response to 5 sec applications of either 1 mM STP or 3 mM GABA alone to a cell expressing the α3β3γ2L isoform. The cell was voltage clamped at −50 mV.

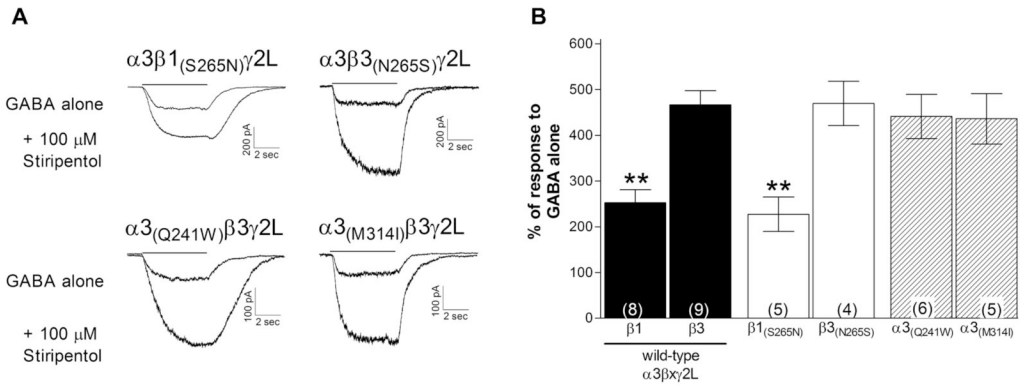

Figure 9. Mutations at sites important for other positive allosteric modulators do not affect STP activity

A. Representative current traces in response to GABA alone or GABA co-applied with 100 μM STP for 5 sec., as indicated by the bar. All cells were voltage-clamped at −50 mV. GABA concentration was 3 μM ($\alpha3_{Q241W,}$ $\alpha3_{M314I}$), 10 μM ($\beta3_{N265S}$) or 30 μM ($\beta1_{S265N}$), representing an $EC_{10–20}$ for each isoform.

B. Bars represent mean potentiation ± SEM for the isoform indicated in response to 100 μM STP with the number of cells given by the number in parentheses. ** indicates a significant difference from α3β3γ2L ($p < 0.01$). The response of receptors containing either mutated β subunit was not significantly different from that of its wild-type counterpart ($p>0.05$).

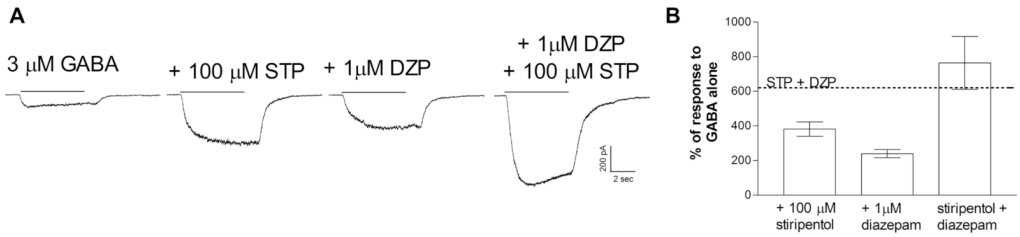

Figure 10. Co-administration of STP and diazepam

A. Currents are shown from cells transfected with α3β3γ2L in response to 3 μM GABA with either 100 μM STP, 1 μM diazepam (DZ) or both STP and DZ co-applied. All responses shown were obtained from the same cell.

B. Bars represent mean ± SEM (N=3) for potentiation by 100 μM STP, 1 μM DZ, or both applied together to the same cell. The dotted line represents the sum of the response to the drugs applied alone.