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Interactions between modulators of the $GABA_A$ receptor: Stiripentol and benzodiazepines

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ABSTRACT

Many patients with refractory epilepsy are treated with polytherapy, and nearly 15% of epilepsy patients receive two or more anti-convulsant agents. The anti-convulsant stiripentol is used as an add-on treatment for the childhood epilepsy syndrome known as severe myoclonic epilepsy in infancy (Dravet syndrome). Stiripentol has multiple mechanisms of action, both enhancing $GABA_A$ receptors and reducing activity of metabolic enzymes that break down other drugs. Stiripentol is typically co-administered with other anti-convulsants such as benzodiazepines which also act through $GABA_A$ receptor modulation. Stiripentol slows the metabolism of some of these drugs through inhibition of a variety of cytochrome P450 enzymes, but could also influence their effects on GABAergic neurotransmission. Is it rational to co-administer drugs which can act through the same target? To examine the potential interaction between these modulators, we transiently transfected HEK-293T cells to produce $\alpha3\beta3\gamma2L$ or $\alpha3\beta3\delta$ recombinant $GABA_A$ receptors. Using whole-cell patch clamp recordings, we measured the response to each benzodiazepine alone and in combination with a maximally effective concentration of stiripentol. We compared the responses to four different benzodiazepines: diazepam, clonazepam, clobazam and norclobazam. In all cases we found that these modulators were equally effective in the presence and absence of stiripentol. The δ-containing receptors were insensitive to modulation by the benzodiazepines, which did not affect potentiation by stiripentol. These data suggest that stiripentol and the benzodiazepines act independently at $GABA_A$ receptors and that polytherapy could be expected to increase the maximum effect beyond either drug alone, even without consideration of changes in metabolism.

1. Introduction

The anti-convulsant stiripentol (Diacomit®) has been investigated for clinical effectiveness in epilepsy for several decades (Trojnar et al., 2005; Chiron, 2007). Although stiripentol did not show greater activity than other common anti-epileptic drugs in clinical trials with adult patients, studies in pediatric populations provided more promising results. The addition of stiripentol to polytherapy reduced the frequency and severity of seizures and status epilepticus in infants and children with a variety of epilepsy syndromes (Perez et al., 1999; Rey et al., 1999; Chiron et al., 2000; Kassaï et al., 2008; Inoue et al., 2009). Stiripentol has since been approved by the European Medicines Agency for the treatment of pharmacoresistant patients with severe myoclonic epilepsy in infancy (Dravet syndrome).

Stiripentol has both direct and indirect anti-convulsant actions. It inhibits a variety of hepatic cytochrome P450 enzymes which metabolize other anti-epileptic drugs (Tran et al, 1997), increasing their duration of action. In addition, stiripentol alone is effective in animal models of acute and chronic seizures (Shen et al., 1992; Trojnar et al, 2005; Luszczki et al., 2010). Recent studies suggest that the mechanism underlying this direct activity is positive allosteric modulation of $GABA_A$ receptors (Quilichini et al., 2006; Fisher, 2009).

The $GABA_A$ receptors are ligand-gated ion channels responsible for fast, inhibitory neurotransmission. Stiripentol acted both pre- and post-synaptically to increase the frequency and slow the decay of GABAergic mIPSCs in hippocampal brain slices (Quilichini et al., 2006). In studies with recombinant $GABA_A$ receptors, stiripentol increased the response in a subunit-dependent manner, with greatest effectiveness at receptors containing an α3 subunit (Fisher, 2009). This subunit is one of the predominant α subtypes in the developing brain (Laurie et al., 1992), which may explain stiripentol's greater clinical efficacy in childhood epilepsy syndromes. Stiripentol was also highly active at δ-containing receptors. These benzodiazepine-insensitive receptors are located extrasynaptically where they produce a tonic current in response to ambient GABA (Belelli et al, 2009).

Stiripentol is approved only for use as add-on therapy, and as such will always be co-administered with another anti-epileptic drug. In many cases, the co-therapy includes a drug targeting the $GABA_A$ receptors, such as the 1,4- or 1,5-benzodiazepines. While a number of

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1,4-benzodiazepines, including diazepam and clonazepam, are widely used, the only 1,5-benozodiazepine used clinically for epilepsy is clobazam (Ng and Collins, 2007). Interestingly, much of the anti-convulsant activity of clobazam may be mediated through its active metabolite, norclobazam (N-desmethyl-clobazam) (Kinoshita et al., 2007). Stiripentol greatly increases the plasma levels of norclobazam by slowing hydroxylation through CYP2C19 (Giraud et al., 2006).

Stiripentol was initially considered for polytherapy because of its action on metabolic enzymes. With the new understanding of its activity at $GABA_A$ receptors, the potential for interactions between stiripentol and other modulators at this target becomes a concern. Therefore, we examined the effect of co-application of stiripentol with benzodiazepines on the activity of recombinant $GABA_A$ receptors to determine if these modulators interacted at the level of the receptor or if their actions were independent.

2. Materials and methods

2.1. Transfection of HEK-293T cells

Full-length cDNAs for rat $GABA_A$ receptor subunits in pCMV expression vectors were transiently transfected into the human HEK-293T cell line. HEK-T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 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 encoding $GABA_A$ receptor subunit cDNAs were added to the cells in 1:1:1 ratios of 2 μg each(α:β:γ or α:β:δ). To identify positively transfected cells, 1 μg of the plasmid pHook™-1 (Invitrogen, San Diego, CA) was also included. Following a 4–6 h 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 s. The selection procedure for pHook expression was performed 44–52 h later. 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 h later.

2.2. 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 chloride equilibrium potential near 0 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 <50 ms (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 (stiripentol, benzodiazepines) on the day of the experiment. Stiripentol was provided by Biocodex (Beauvais, France) and norclobazam was synthesized by Chemtos (Austin, TX). Diazepam, clonazepam and clobazam were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. 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 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 peak current elicited by the response to GABA alone or GABA + 100 μM stiripentol. Statistical tests were performed using the Instat program (Graphpad). Differences between treatments were determined with a Student's t-test with a minimum P value for significance of 0.05.

3. Results

3.1. 1,4- and 1,5-benzodiazepines modulate recombinant α3β3γ2L $GABA_A$ receptors with differing efficacy and potency

Because stiripentol is most effective at recombinant $GABA_A$ receptors that contain the α3 subunit (Fisher, 2009), all experiments were conducted with α3β3γ2L or α3β3δ receptor isoforms. We first compared the ability of the benzodiazepines to modulate the response of recombinant α3β3γ2L receptors to a sub-maximal concentration (EC_{10-20}) of GABA.

3.1.1. Diazepam and clonazepam

Diazepam strongly enhanced the response of α3β3γ2L receptors to 3 μM GABA, with an average EC_{50} of 59.4 ± 19.0 nM and maximum potentiation of 508.4 ± 28.4% (N = 5) (Fig. 1). Clonazepam was also a very potent modulator of these receptors, with an average EC_{50} of 89.8 ± 22.5 nM (N = 4). However, clonazepam had lower efficacy than diazepam, with an average maximum potentiation of 262.7 ± 22.3% (N = 4).

3.1.2. Clobazam and norclobazam

Few studies have examined the action of clobazam or norclobazam on recombinant $GABA_A$ receptors. We found that at α3β3γ2L receptors clobazam and norclobazam had lower potency than diazepam, but were similar to one another, with average EC_{50} values of 493.0 ± 63.2 nM (N = 4) and, 554.7 ± 209.2 nM (N = 4) respectively (Fig. 1). The maximum potentiation in response to norclobazam was substantially lower than that of clobazam, with an average of 270.5 ± 24.8% (N = 4), compared to 487.3 ± 38.7% (N = 4) for clobazam. This is consistent with the lower *in vivo* activity associated with the metabolite compared to the parent drug (Brogden et al., 1980).

3.2. Co-application with stiripentol

Since stiripentol is always used clinically in combination with other anti-epileptic drugs, it is important to characterize potential interactions at the $GABA_A$ receptors. Therefore, we examined the ability of diazepam, clonazepam, clobazam or norclobazam to increase the GABA-activated current amplitude of α3β3γ2L receptors in the presence of a maximally effective concentration of stiripentol (100 μM) (Fig. 2).

3.2.1. Diazepam and clonazepam

The EC_{50} for stiripentol modulation of α3β3γ2L receptors was reported to be ~25 μM with a peak potentiation at concentrations near