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3α-Hydroxy-3β-trifluoromethyl-5α-pregnan-20-one (Co 2-1970): A Partial Agonist at the Neuroactive Steroid Site of the γ-Aminobutyric acid$_A$ Receptor

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SUMMARY

Neuroactive steroids bind to a unique site on the γ-aminobutyric acid$_A$ (GABA$_A$) receptor complex and allosterically modulate the binding of convulsant ([^{35}S]*t*-butylbicyclophosphorothionate, [^{35}S]TBPS), GABA ([^{3}H]muscimol), and benzodiazepine ([^{3}H]flunitrazepam) site ligands. In rat cortical membranes, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) is a full agonist at the steroid site, inhibiting 96% of specific [^{35}S]TBPS binding and enhancing [^{3}H]flunitrazepam and [^{3}H]muscimol binding 95% and 69% above control levels, respectively. In contrast, the synthetic steroid 3α-hydroxy-3β-trifluoromethyl-5α-pregnan-20-one (Co 2–1970) has limited efficacy for modulating the binding of [^{35}S]TBPS (44% inhibition), [^{3}H]flunitrazepam (41% enhancement), and [^{3}H]muscimol (<10% enhancement). In competition experiments, Co 2–1970 (10 μM) reduced the apparent potency of 3α,5α-P by 7–17-fold for modulating the binding of these radioligands in rat cortical membranes, suggesting that it has partial agonist properties. Because cortical membranes contain a heterogeneous population of receptors, Co 2–1970 was examined in recombinant GABA$_A$ receptors stably expressed in human embryonic kidney 293 cells. Co 2–1970 inhibited [^{35}S]TBPS binding with limited efficacy (39–65% inhibition) in the five receptor combinations examined and, at 10 μM, reduced the apparent potency of 3α,5α-P 57-fold for inhibiting [^{35}S]TBPS binding to α1β1γ2L receptors. To verify these findings functionally, the effects of 3α,5α-P and Co 2–1970 were examined electrophysiologically in *Xenopus* oocytes expressing α1β1γ2L receptors. Co 2–1970 showed limited efficacy potentiation of GABA-evoked chloride currents relative to 3α,5α-P (28% and 86% of the GABA maximum current, respectively). Moreover, Co 2–1970 produced a concentration-dependent antagonism of the 3α,5α-P-induced potentiation that was associated with a reduction in the apparent affinity of 3α,5α-P (11-fold at 10 μM Co 2–1970). Taken together, these data indicate that Co 2–1970 is a partial agonist at the neuroactive steroid site associated with GABA$_A$ receptors.

Mammalian GABA$_A$ receptors are ligand-gated ion channels comprised of hetero-oligomeric assemblies of polypeptide subunits (1). Biochemical and cloning studies have shown that there are four subunit classes, denoted α, β, γ, and δ, most of which have multiple variants (1). These subunits and variants have distinct patterns of distribution in brain (2, 3), suggesting that different subtypes of GABA$_A$ receptors are selectively involved in different aspects of brain function.

GABA$_A$ receptors are allosterically modulated by a structurally diverse group of ligands. These include benzodiazepines, β-carbolines, and related compounds (4); neuroactive steroids (5, 6); barbiturates (7); picrotoxinin, cage convulsants (e.g., TBPS), and insecticides (8); phenylethenyltriazoles, such as loreclezole (9); volatile and nonvolatile anesthetics (10); ethanol (11); and some nonsteroidal anti-inflammatory drugs (e.g., mefenamic acid) (12). Distinct allosteric modulatory sites have been identified for benzodiazepines, neuroactive steroids, barbiturates, the convulsants, and loreclezole (4–9). Mechanisms of action for anesthetics, nonsteroidal anti-inflammatory drugs, ethanol, and other types of modulators are less certain. Allosteric sites on GABA$_A$ receptors have proved to be a valuable target for the development of therapeutically useful anxiolytics, anticonvulsants, and sedative/hypnotics (13–15), and designing modulators with novel characteristics is a well established strategy for finding improved therapies in these areas. Subtype selectivity and limited efficacy are features that have

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ABBREVIATIONS: GABA, γ-aminobutyric acid; Co 2–1970, 3α-hydroxy-3β-trifluoromethyl-5α-pregnan-20-one; TBPS, *t*-butylbicyclophosphorothionate; 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

been exploited in the benzodiazepine area for the design of drugs with improved or novel pharmacological and therapeutic profiles (4).

The pharmacology of steroid modulatory sites on $GABA_A$ receptors is currently less well developed than that for the benzodiazepines (5, 6). Several lines of evidence suggest that steroids show subtype selectivity in their interactions with $GABA_A$ receptors. In binding studies with native receptors, neuroactive steroids show brain regional variation for allosteric modulation (16) and differential selectivity for putative receptor subtypes (17). Similarly, regional variation (18) and two-component potentiation (19) of $^{36}Cl^-$ uptake are suggestive of receptor heterogeneity. On the other hand, subtype-dependent differences in neuroactive steroid efficacy but not affinity have been observed in electrophysiological and binding studies with cloned receptors (20, 21).

Several steroids have been identified that have limited efficacy for allosteric modulation of [^{35}S]TBPS and [^{3}H]flunitrazepam binding in rat brain (22–24), suggesting that ligands interact with the neuroactive steroid site with varying levels of intrinsic efficacy. However, this low efficacy modulation in brain membranes, containing a variety of receptor subtypes, may involve partial agonism, subtype selectivity, multiple sites of action, or any combination of these effects. Early studies with 5α-pregnan-3α,20α-diol provided evidence for partial agonism in $^{36}Cl^-$ flux assays (25), although recent work suggests that this progesterone metabolite may be selective for a subclass of neuroactive steroid binding sites (17). In addition to partial agonism, evidence that 3β-hydroxy-5β-pregnan-20-one (26) and Δ16-alphaxalone (27) antagonize the actions of neuroactive steroids suggest that certain structural modifications can result in antagonists for steroid modulatory sites. Nevertheless, these putative antagonists have not been evaluated functionally on recombinant receptors; therefore, their mechanism of antagonist action remains speculative. With respect to inverse agonism, pregnenolone sulfate has been shown to inhibit GABA responses (21, 28), although current evidence suggests that this inhibition is mediated by a site distinct from that involved in the positive modulation (29).

To further explore the structure-activity relationship for steroids at the $GABA_A$ receptor, a series of 3β-substituted steroids was synthesized and assayed for inhibition of [^{35}S]TBPS binding in rat brain membranes (24). Co 2–1970 (Fig. 1) was found to have low efficacy in the [^{35}S]TBPS binding assay, in contrast to the full inhibitor 3α,5α-P (Fig. 1). In the current study, we report a detailed characterization of Co 2–1970 and provide evidence that it is a partial agonist for the neuroactive steroid site.

Materials and Methods

Drugs

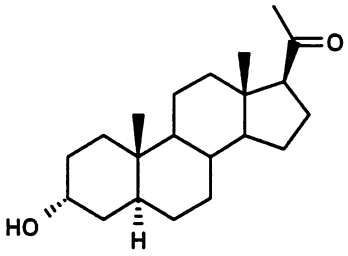
Co 2–1970 and 3α,5α-P were synthesized as previously described (24).

Stable Cell Lines

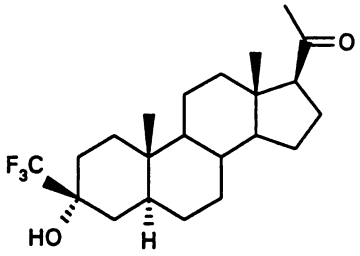
Stable expression of $GABA_A$ receptor subunits. Human α1, α2, α3, β1, and γ2L $GABA_A$ receptor subunits (obtained from Dr. Peter Seeburg, University of Heidelberg, Heidelberg, Germany) were cloned into mammalian expression vectors containing the constitutive enhancer/promoter of the immediate early gene of human cytomegalovirus, pCDM8 (InVitrogen), or pcDNA1 (InVitrogen). The 1464-base pair nucleotide fragment (1389 of coding region and 75 of linker region) covering the 459-amino acid coding region of the human α5 subunit was cloned through reverse transcription and PCR, with primers flanking the putative first and last amino acids of the human α5 sequence. The PCR primer sequences were derived from the reported human α5 sequence (30). The human α5 cDNA was then subcloned into the pCDNAI vector (InVitrogen) for mammalian expression under the cytomegalovirus mammalian expression promoter. The α5 cDNA was sequenced on both strands through the dideoxy chain termination reaction (Sequenase II, Amersham). The amino acid sequence derived from this cDNA was identical to the amino acid sequence previously reported (31). The human β2 cDNA was obtained from the rat β2 sequence (32) by mutating the codon for amino acid 347 from asparagine to serine (33) and then ligating into the pcDNA1 expression vector.

All plasmid DNA for transfection was prepared through two-cycle cesium chloride gradient centrifugation. The transfection follows the protocol reported previously (34). One day before transfection, the human embryonic kidney 293 cells (CRL 1573; American Type Culture Collection) grown to confluency in a 110-cm^2 flask were diluted 1:15 and split into 10- or 15-cm-diameter culture dishes. On the day of transfection, 7–10 μg of each subunit cDNA in various combinations along with the expression plasmid pY3 (0.1 μg), containing the gene that confers resistance to the antibiotic hygromycin B, was diluted with distilled water to 250 μl and mixed with 250 μl of 0.5 M calcium chloride and 500 μl of 2× *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 6.95). The mixture was then incubated at room temperature for 15 min, added to the culture dish, and incubated in a 3% CO_2 and 35° humidified incubator for 16–20 hr. The cells were then washed with 3 ml serum-free Dulbecco's modified Eagle medium (Life Technologies) and incubated in a 5% CO_2 and 37° humidified incubator with Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

Selection was started 48 hr later by replacing the medium with complete medium plus 200 μg/ml hygromycin B (Calbiochem). After 2 weeks, resistant colonies were trypsinized in cloning cylinders and transferred to 12-well plastic plates. Individual cell lines were expanded and maintained in the same medium.



3α-hydroxy-5α-pregnan-20-one
3α,5α-P



3α-hydroxy-3β-trifluoromethyl-5α-pregnan-20-one
Co 2-1970

Fig. 1. Chemical structures of the naturally occurring neuroactive steroid 3α,5α-P and the 3β-trifluoromethyl derivative Co 2–1970.

Analysis of subunit incorporation. Cell lines were analyzed for the presence of GABA receptor gene mRNAs through reverse transcription of total RNA followed by PCR. Total RNA was prepared according to the acid/phenol method (35). The reverse transcription analysis for human $GABA_A$ subunit mRNA transcription has been described previously (36). The PCR parameters were 94° for 5 min, 55° for 1 min, and 72° for 1.5 min for the first cycle; followed by 40 cycles of 94° for 1 min, 55° for 1 min, and 72° for 1.5 min; and followed by an 8-min extension at 72°. PCR products were analyzed through electrophoresis in 1–2% agarose gels. Cell lines that were positive for the α, β, and γ subunit mRNA transcripts were then analyzed for the presence of $GABA_A$ receptor complexes by their ability to bind [^{35}S]TBPS (see below). Functional incorporation of the γ subunit was determined by [^{3}H]flunitrazepam binding for α1-, α2-, and α3-containing receptors or by [^{3}H]Ro15–4513 binding for α5-containing receptors as described previously (37).

Membrane Preparation

Stable cell line membranes. Cells were harvested by decanting the incubation media and replacing it with 1 ml of 10× trypsin/EDTA solution (Life Technologies). After 5-min incubation with gentle agitation, 9 ml of serum-containing media was added, and the cells were released from the flask by gently pipetting the culture medium up and down. The culture medium was then removed through low speed centrifugation at 1000 × *g* for 10 min and rinsed twice with cold 200 mM NaCl/50 mM Na-K phosphate, pH 7.4, buffer (PBS). Cell membranes were disrupted with a Polytron (Brinkmann) at setting 10 for 20 sec. The cell homogenate was centrifuged at 9000 × *g* for 20 min, and the pellet was rinsed once before resuspension with cold PBS in the desired volume for the binding assay.

Rat brain cortical membranes for the TBPS and flunitrazepam assays. Rat cortical membranes were prepared as previously described (22, 23). Briefly, cortices were rapidly removed after decapitation of carbon dioxide-anesthetized male Sprague-Dawley rats (200–250 g), homogenized in 10 volumes of ice-cold 0.32 M sucrose with a glass/Teflon homogenizer, and centrifuged at 1500 × *g* for 10 min at 4°. The resultant supernatants were centrifuged at 10,000 × *g* for 20 min at 4° to obtain the P2 pellets. The P2 pellets were resuspended in PBS and centrifuged at 10,000 × *g* for 10 min at 4°. This washing procedure was repeated twice, and the final pellets were resuspended in 10 volumes of PBS.

Rat brain cortical membranes for the muscimol assay. Rat cortical membranes were prepared by the recently described method allowing for high levels of [^{3}H]muscimol binding and its modulation by neuroactive steroids (38). After the homogenization of rat cortices and first centrifugation step described above, the resultant supernatants were centrifuged at 20,000 × *g* for 20 min at 4° to obtain the P2 pellets. The P2 pellets were resuspended in 10 volumes of water and centrifuged for 20 min at 8000 × *g*. The supernatant and white buffy layer were centrifuged at 250,000 × *g* for 20 min. The resultant pellet was washed once more with water and once with Na-free buffer (100 mM KCl/40 mM KH_2PO_4, pH 7.4). This pellet was resuspended in 35 ml Na-free buffer, incubated at 37° for 30 min, and then centrifuged at 31,000 × *g* for 20 min. The final pellet was resuspended in 10 volumes of Na-free buffer for the binding assay.

Radioligand Binding Assays

[^{35}S]TBPS binding assay. The [^{35}S]TBPS assay was performed as described previously (22, 23). Aliquots (100 μl) of the brain membrane suspension (300–350 μg of protein) or cell membrane suspension (150–200 μg) were incubated with 2 nM [^{35}S]TBPS (60–100 Ci/mmol; New England Nuclear) and 5-μl aliquots of Co 2–1970 or 3α,5α-P dissolved in DMSO (final, 0.5–1%) in the presence of GABA (Sigma Chemical Co.) at the concentration stated in the figure legends. The incubation was brought to a final volume of 1.0 ml with PBS. Nonspecific binding was determined in the presence of 2 μM unlabeled TBPS (obtained from Dr. Christopher Palmer, Ricerca, Inc., Painesville, OH) and ranged from 15% to 25% for both rat brain and stable cell line membranes. After a 90-min incubation at room temperature, the assays were terminated by filtration through glass fiber filters (Schleicher and Schuell, No. 32) with a cell harvester (Brandel) and rinsed three times with ice-cold buffer. Filter-bound radioactivity was measured with liquid scintillation spectrometry. Protein was determined with a modified Lowry assay (39).

[^{3}H]Flunitrazepam binding assay. The [^{3}H]flunitrazepam assay was identical to the [^{35}S]TBPS assay except that the membranes were incubated with 1 nM [^{3}H]flunitrazepam (74–84 Ci/mmol; NEN) in the presence of 1 μM GABA. The steroids were added in 5 μl 2-methoxyethanol (final, 0.5–1%). Nonspecific binding was determined in the presence of 1 μM clonazepam (Sigma) and ranged from 2% to 5%.

[^{3}H]Muscimol binding assay. The [^{3}H]muscimol assay has recently been described (38). Aliquots (100 μl) of the membrane suspensions were incubated with 5 nM [^{3}H]muscimol (10.1 Ci/mmol; NEN) and 5-μl aliquots of Co 2–1970 or 3α,5α-P dissolved in DMSO (final, 0.5–1%) in a final volume of 1.0 ml Na-free buffer. Nonspecific binding was determined in the presence of 1 mM GABA and ranged from 5% to 10%. After a 60-min incubation at 4° in the dark, the assays were terminated as described above and counted after the filters were allowed to stand overnight in cocktail.

Electrophysiology

Preparation of RNA. RNA was transcribed from *Not*I-linearized cDNA with a methylated guanine cap analogue and T7 RNA polymerase, as described in the mMessage mMachine protocol (Ambion). cRNA was diluted to 1 μg/μl with diethylpyrocarbonate-treated water and stored in 1–2 μl aliquots at −80° until injection.

Receptor expression in *Xenopus* oocytes. Ovarian lobes were surgically removed from mature female *Xenopus laevis* and stored in Barth's medium containing 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 5 mM HEPES, pH 7.4, with 0.1 mg/ml gentamycin sulfate (28). Follicle-enclosed oocytes were manually dissected from the ovary. The inner ovarian epithelium, theca, and most of the follicular layer were removed enzymatically by treatment with collagenase (0.5 mg/ml for ~1 hr; Boehringer Mannheim) and brief vortexing. Immediately before injection, stock cRNAs were mixed and diluted 10-fold in water. Oocytes were microinjected with mixtures containing ~1 ng of cRNA encoding each $GABA_A$ receptor subunit.

Electrical recordings and drug application. Membrane current responses were recorded with a conventional two-electrode voltage clamp in frog Ringer's solution containing 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4. Diverging from previously described procedures (28), oocytes were placed in a ~5 ml recording chamber lined with a 0.5–0.7-mm mesh nylon screen (Spectrum Medical Industries). The oocyte was held to the mesh by the downward pressure of the electrodes. Drug and wash solutions were applied directly to the oocyte with a scaled-up version of the microcapillary "linear array" system commonly used to apply drugs to cultured mammalian neurons. For the oocyte recordings, three 104-mm Drummond Microcaps (volume, 200 μl; o.d., ~2 mm) were mounted to a standard micromanipulator. The tips of the capillaries were positioned 2–3 mm from the oocyte at an angle of ~45° and moved laterally with the course right-left adjustment. Each capillary had a separate reservoir. Flow rates (5–10 ml/min) were sufficient to establish a "liquid filament" without disturbing the preparation. To ensure that the solution around the oocyte was completely dominated by the flow from a single capillary, it was convenient to separate the "active" capillaries by 1–2 capillary diameters; the use of directly adjacent tubes resulted in sporadic cross-talk between solutions. When changing from one tube to another, there were, therefore, transient (~100 msec) exposures to the background bathing medium. To provide a background flushing flow, the chamber was perfused at a low rate (1–5 ml/min) with Ringer's solution. Separation of active capillaries was not a problem for the present

experiments, which were basically steady state modulation measurements, but should be avoided for any study involving response kinetics.

The scaled-up linear array system allows for drug exposures that can be limited to ~1 sec. This is useful when dealing with desensitizing responses. Drugs can also be applied quite rapidly with the preestablished liquid filament. For example, sampled rise times (10–90%) for responses to 10 mM GABA in oocytes expressing $\alpha1\beta1\gamma2L$ receptors ranged from 200 to 850 msec (mean, 420 ± 120 msec; six experiments). Values were similar for binary $\alpha1\beta1$ receptors. Variability was mainly between oocytes as opposed to between repeated applications to the same cell. This suggests that the limiting factor in applying drugs to the oocyte surface is not the speed of the drug perfusion system but rather the complexity of the surface itself and the permeability of the vitelline envelope (40).

Steroid solutions and vehicle controls. 3α,5α-P and Co 2–1970 were first made up at 10 mM in DMSO. Serial DMSO dilutions were made to generate stocks over the range of 0.001–10 mM, which were stored at room temperature for a period of a few days. Bench life of 10 mM steroid stocks was limited by recrystallization in the DMSO. Ringer solutions were made immediately before use through 1000–3000-fold dilutions of stocks. Before stock dilution, DMSO was adjusted to 0.3% by volume in all test solutions. This procedure minimized the risk of steroid precipitating in the saline. At concentrations of ≤0.3%, DMSO had little or no effect on GABA responses in oocytes expressing $\alpha1\beta1\gamma2L$ subunit combinations. For example, in one batch of cells exposed to 0.3% DMSO, the current (expressed as a fraction of control) was 1.01 ± 0.01 (four experiments) for 5% responses and 1.02 ± 0.01 (seven experiments) for maximum GABA responses.

Data Analysis

The binding data were fit to the sigmoidal equation (Equation 1), where Y is the percent specific bound, A is the bottom plateau, B is the top plateau, X is log concentration, C is the log of the IC_{50} (or EC_{50}), and D is the Hill coefficient (n_H) (Prism, GraphPad):

$$Y = A + \frac{(B - A)}{1 + 10^{(C-X)\cdot D}} \quad (1)$$

The maximal extent of inhibition (I_{max}) and enhancement (E_{max}) are defined as the absolute difference of A and B. D is negative or positive for inhibition or enhancement curves, respectively. The data were fit to a partial ($A > 0$) instead of a full ($A = 0$) inhibition model if the sum of squares was significantly lower by F test. The Hill coefficient was allowed to vary from unity if the sum of squares was significantly lower by F test.

For electrophysiology, GABA concentration-response data were fit with use of the logistic equation (Equation 2), where EC_{50} is the concentration of GABA that elicits half-maximal responses, n is the slope, and FR is the current expressed as a fraction of the maximum GABA response (Origin, Microcal):

$$FR = \frac{1}{1 + (EC_{50}/[\text{agonist}])^n} \quad (2)$$

Steroid concentration-modulation data were also fit to the logistic equation. For these curves, all currents were scaled with respect to the maximum GABA response, and initial and final values were not fixed. Data in the text are given as mean ± standard error to two significant figures.

Results

Co 2–1970 behaves as a partial agonist in allosteric radioligand binding assays with rat brain cortical membranes. In the presence of 5 μM GABA, Co 2–1970 is a limited efficacy inhibitor of [^{35}S]TBPS binding in rat brain cortical membranes in contrast to the full inhibitor 3α,5α-P (Fig. 2A). In the absence of added GABA to well-washed P2 membranes, Co 2–1970 is essentially inactive. In contrast, 3α,5α-P retains full efficacy for inhibition of [^{35}S]TBPS binding in the absence of GABA, although it has lower potency than in the presence of GABA (17). Similar to Co 2–1970, the

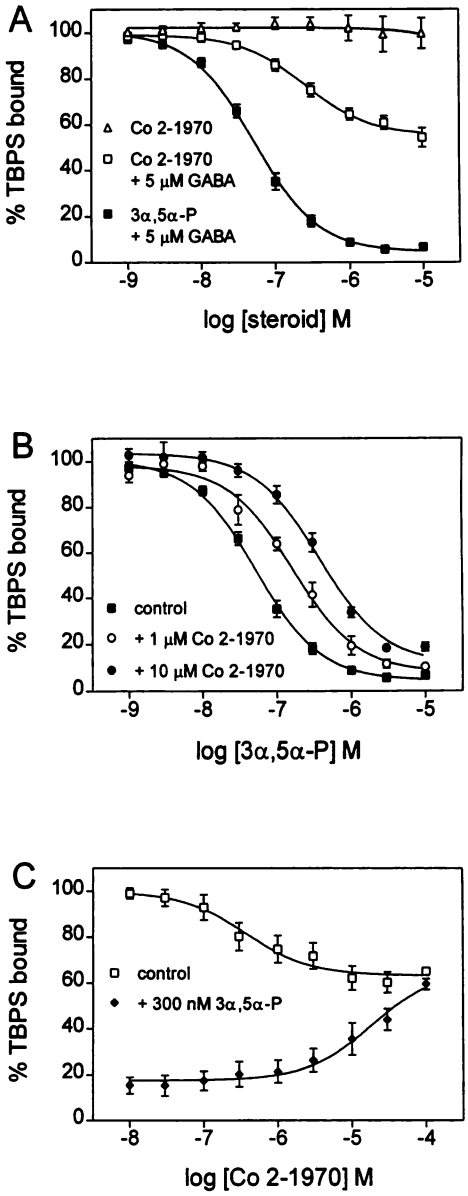


Fig. 2. Co 2–1970 is a limited efficacy inhibitor of [^{35}S]TBPS binding in rat brain cortical membranes and antagonizes the inhibition by the full inhibitor 3α,5α-P. A, Limited efficacy concentration-effect curve for Co 2–1970 (IC_{50}, 266 ± 43 nM; I_{max}, 44 ± 4%; n_H = 1.0; seven experiments) compared with the full inhibitor 3α,5α-P (IC_{50}, 53 ± 6 nM; I_{max}, 96 ± 1%; n_H = 1.0; six experiments) in the presence of 5 μM GABA. Co 2–1970 is inactive in the absence of added GABA (three experiments). B, 3α,5α-P inhibition of [^{35}S]TBPS binding in the absence and presence of 1 μM (IC_{50}, 164 ± 14 nM; I_{max}, 90 ± 5%; n_H = 1.0) or 10 μM (IC_{50}, 373 ± 36 nM; I_{max}, 92 ± 4%; n_H = 1.0) Co 2–1970, indicating a concentration-dependent decrease in the apparent potency of 3α,5α-P (three experiments). GABA concentration was 5 μM. C, Concentration-dependent antagonism of the 300 nM 3α,5α-P inhibition of [^{35}S]TBPS binding by Co 2–1970, showing that Co 2–1970 can reverse the inhibition by 3α,5α-P up to control levels (three experiments). GABA concentration was 5 μM.

TABLE 1

Comparison of the modulatory potency of Co 2–1970 with its potency as an antagonist of 3α,5α-P modulation calculated with the Gaddum-Schild relationship

Assay	Receptors	Co 2–1970 IC_{50} or EC_{50}	Antagonism [Co 2–1970]	Dose ratio[a]	Co 2–1970 K_i or K_b[b]
		nM	μM		nM
TBPS	Rat cortex	266 ± 43	1	3.1	480
			10	7.0	1660
TBPS	α1β1γ2L	98 ± 10	10	57	180
Flunitrazepam	Rat cortex	358 ± 25	10	17	640
Muscimol	Rat cortex	IA[c]	10	21	490
Electrophysiology	α1β1γ2L	220 ± 20	1	3.3	440
			10	11	1030

[a] Dose ratio = $\frac{3\alpha,5\alpha\text{-P } IC_{50} \text{ or } EC_{50} \text{ in the presence of Co 2–1970}}{\text{Control } 3\alpha,5\alpha\text{-P } IC_{50} \text{ or } EC_{50}}$

[b] K_i or K_b = $\frac{[\text{Co 2–1970}]}{\text{Dose ratio} - 1}$

[c] IA = inactive.

limited efficacy inhibitor 5α-pregnan-3α,20α-diol is inactive in the [^{35}S]TBPS assay in the absence of GABA (17). To test the potential of Co 2–1970 to antagonize a full efficacy neuroactive steroid, concentration-effect curves for 3α,5α-P were determined in the presence of increasing concentrations of Co 2–1970. As shown in Fig. 2B, Co 2–1970 decreases the apparent potency of 3α,5α-P as an inhibitor of [^{35}S]TBPS binding, with a 3.1- and 7.0-fold shift at 1 and 10 μM Co 2–1970, respectively (Table 1). In the presence of a fixed concentration of 3α,5α-P resulting in >80% inhibition of [^{35}S]TBPS binding, increasing concentrations of Co 2–1970 decrease the level of inhibition by 3α,5α-P to approximately control levels, indicating a decrease in the apparent efficacy of the full inhibitor (Fig. 2C).

Co 2–1970 has a partial agonist profile with [^{3}H]flunitrazepam as radioligand. Co 2–1970 produces limited efficacy enhancement of [^{3}H]flunitrazepam binding compared with 3α,5α-P in the presence of 1 μM GABA (Fig. 3A). Co 2–1970 (10 μM) decreases the apparent potency of 3α,5α-P for enhancement of [^{3}H]flunitrazepam binding by 17-fold (Fig. 3B and Table 1). Thus, Co 2–1970 exhibits limited efficacy and antagonizes the modulation induced by a full agonist neuroactive steroid in allosteric binding assays measuring coupling to both convulsant and benzodiazepine binding sites.

In contrast to results obtained with [^{3}H]flunitrazepam and [^{35}S]TBPS, Co 2–1970 is virtually inactive in modulating [^{3}H]muscimol binding. Thus, Co 2–1970 enhances binding <10% at concentrations of ≤100 μM, whereas 3α,5α-P enhances [^{3}H]muscimol binding to >160% of control levels (Fig. 4A). Although the slope of the 3α,5α-P enhancement curve is shallow (n_H = 0.75 ± 0.15), a clear two-component enhancement of [^{3}H]muscimol binding was not observed, in contrast with previously reported data (38). Although it does not significantly modulate [^{3}H]muscimol binding, Co 2–1970 (10 μM) decreases the apparent potency of 3α,5α-P for enhancing [^{3}H]muscimol binding by 12-fold, indicating that it does have affinity for the neuroactive steroid site under these conditions (Fig. 4B and Table 1).

Co 2–1970 behaves as a partial agonist in allosteric radioligand binding assays with recombinant $GABA_A$ receptors. Co 2–1970 is a limited efficacy inhibitor of [^{35}S]TBPS binding to membranes prepared from human embryonic kidney 293 cells expressing recombinant receptors containing four different α variants and two different β variants (Fig. 5A and Table 2). Co 2–1970 displayed differential potency in these recombinant receptors with the following rank order: α5β2γ2L ~ α1β1γ2L > α2β2γ2L ~ α3β2γ2L > α1β2γ2L (Table 2). This steroid was >10-fold more potent as an inhibitor of [^{35}S]TBPS binding in α5β2γ2L and α1β1γ2L than in α1β2γ2L combinations. Co 2–1970 has lower efficacy in cells expressing α1β2γ2L and α2β2γ2L complexes (I_{max} = 39–43%) than in cells expressing α1β1γ2L, α3β2γ2L, and α5β2γ2L complexes (I_{max} = 54–65%) (Fig. 5A and Table 2). Nevertheless, Co 2–1970 displays low efficacy inhibition of [^{35}S]TBPS binding relative to the full inhibitor 3α,5α-P in all cell lines tested (Fig. 5B and Table 2). The interaction of Co 2–1970 with 3α,5α-P was explored further in a stable cell line expressing α1β1γ2L receptor complexes because Co 2–1970 has high potency at this receptor and because it provided consistency with electrophysiological experiments (see below). As in rat cortical membranes, Co 2–1970 (10 μM) antagonizes the 3α,5α-P-induced inhibition of [^{35}S]TBPS binding in a stable cell line expressing α1β1γ2L receptor complexes, producing a 57-fold reduction in the apparent potency of 3α,5α-P (Fig. 6).

Electrophysiological characterization of α1β1γ2L receptors expressed in *Xenopus* oocytes. Oocytes injected with a mixture of α1, β1, and γ2L cRNAs (~1 ng of each) showed robust expression of functional $GABA_A$ receptors. The binary subunit combinations α1β1 and β1γ2L generally expressed less strongly, and α1γ2L and β1 alone expressed very weakly. For example, responses to 10 mM GABA (maximum responses) in oocytes from one frog were: α1β1γ2L, 1500 ± 40 nA (six experiments); α1β1, 600 ± 130 nA (eight experiments); β1γ2L, 220 ± 40 nA (seven experiments); and α1γ2L, 1 ± 0.5 nA (six experiments).

In oocytes expressing α1β1γ2L, the response to 10 mM GABA was not blocked by 100 μM $ZnCl_2$ (fractional response, 1.01 ± 0.04; four experiments) (41) and was not increased by the coapplication of 300 μM pentobarbital (fractional response, 1.01 ± 0.01; four experiments). In contrast, 10 mM GABA responses were strongly blocked by 100 μM $ZnCl_2$ in oocytes expressing α1β1 subunit combinations (fractional response, 0.15 ± 0.04; eight experiments) and were markedly increased by 300 μM pentobarbital in oocytes expressing β1γ2L (fractional response, 1.7 ± 0.2; seven experiments). Collectively, these results indicated that expression of α1β1γ2L subunits generated $GABA_A$ receptors with hetero-

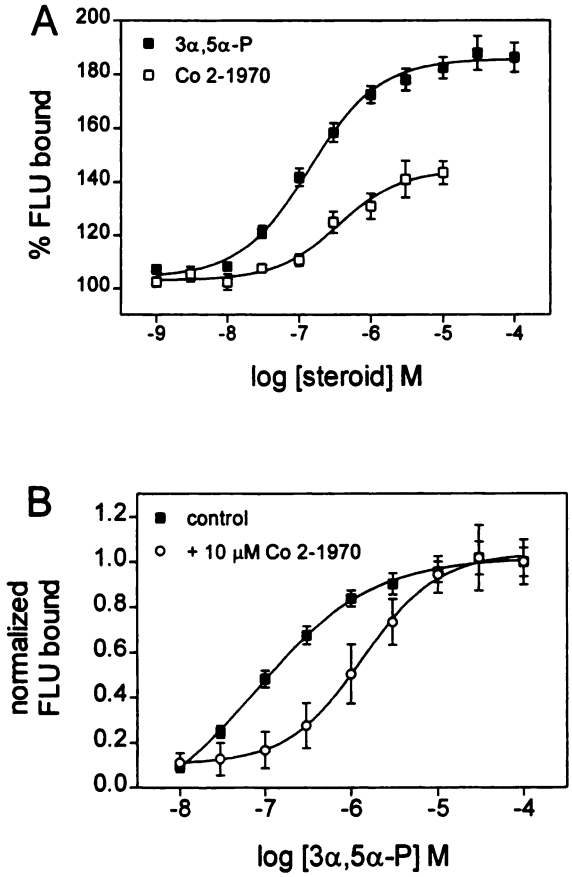


Fig. 3. Co 2–1970 enhances [^{3}H]flunitrazepam (*FLU*) binding in rat brain cortical membranes with limited efficacy and antagonizes the enhancement by 3α,5α-P. A, Limited efficacy concentration-effect curve for Co 2–1970 (EC_{50}, 358 ± 25 nM; E_{max}, 41 ± 4%; n_H = 1.0; three experiments) compared with the full efficacy neuroactive steroid 3α,5α-P (EC_{50}, 92 ± 15 nM; E_{max}, 95 ± 10%; n_H 0.77 ± 0.08; five experiments). B, 3α,5α-P enhancement of [^{3}H]flunitrazepam binding in the absence and presence of 10 μM Co 2–1970 (EC_{50}, 1530 ± 305 nM; E_{max}, 33 ± 1%; n_H = 1.0; five experiments) showing the decrease in the apparent potency of 3α,5α-P. Data are normalized to eliminate the enhancement by Co 2–1970 alone for clarity.

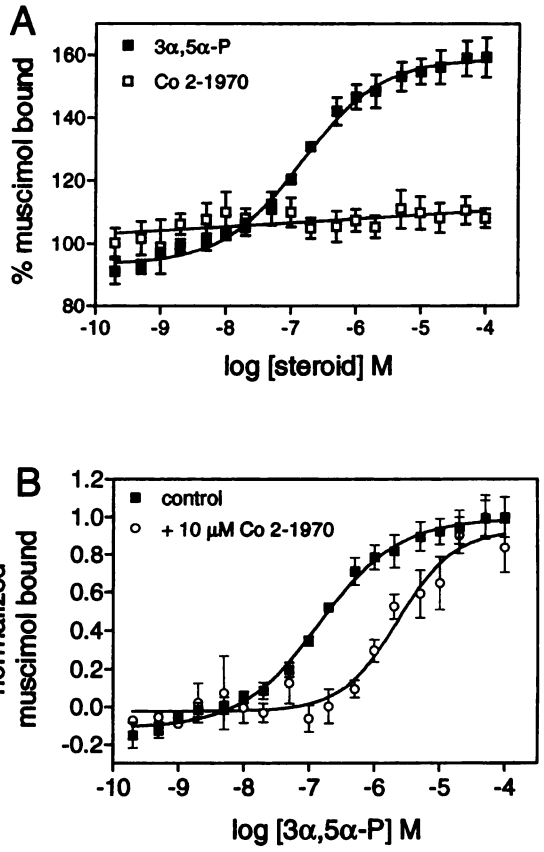


Fig. 4. Co 2–1970 is essentially inactive in the [^{3}H]muscimol binding assay in rat brain cortical membranes and antagonizes the enhancement by 3α,5α-P. A, Lack of modulation by Co 2–1970 (E_{max}, <10%; four experiments; linear regression shown) compared with the concentration-effect curve for the full efficacy neuroactive steroid 3α,5α-P (EC_{50}, 133 ± 27 nM; E_{max}, 69 ± 6%; n_H = 0.75 ± 0.15; five experiments). B, 3α,5α-P enhancement of [^{3}H]muscimol binding in the absence and presence of 10 μM Co 2–1970 (EC_{50}, 2830 ± 750 nM; E_{max}, 40 ± 4%; n_H = 1.0; four experiments) showing the decrease in the apparent potency of 3α,5α-P. Data are normalized for clarity and consistency.

trimeric subunit composition, although the precise subunit stoichiometry was uncertain.

Modulatory effects of steroids were assessed with oocytes expressing α1β1γ2L subunits. In these experiments, maximum GABA responses ranged between 1300 and 3200 nA (mean, 2200 ± 110 nA, 16 experiments). GABA concentration-response curves yielded EC_{50} and slope values of 65 ± 4 μM and 1.4 ± 0.1, respectively (16 experiments, data not shown). The values were typical for oocytes expressing α1β1γ2L subunits and gave no indication of a pharmacologically heterogeneous population of receptors. For measurement of steroid concentration-effect curves, it was first necessary to establish a stable, or near-stable, maximum GABA response. In some cases, responses took up to 1 hr to stabilize. A concentration of GABA was then selected that elicited current that was ~5% of the maximal GABA response. Mean concentrations and values were 10 ± 0.1 μM and 4.9 ± 0.2%, respectively (10 experiments). Modulation was assayed on these responses with 1–2-min preincubations with steroids, followed immediately by a mixture of steroid and GABA (Fig. 7A). Any change in the 10 mM GABA response during the course of the experiment was compensated for by calculating fractional currents against a linear sliding scale.

Co 2–1970 behaves as a partial agonist in potentiating GABA-evoked currents in oocytes expressing recombinant $GABA_A$ receptors. The full efficacy agonist 3α,5α-P caused pronounced increases in GABA-activated currents (Fig. 7, A and B). Maximum potentiation, expressed as a fraction of the response to 10 mM GABA, was 0.86 ± 0.02. Of this, 0.03 ± 0.02 was current activated directly by 3α,5α-P alone (i.e., current elicited during pretreatment with 3α,5α-P). Co 2–1970 also potentiated GABA responses (Fig. 7, A and B). In this case, however, the maximum level of potentiation was only 0.28 ± 0.01. The component activated by Co 2–1970 alone was <0.005. For both 3α,5α-P and Co 2–1970, maximum potentiation of GABA currents was observed at 1–3 μM and began to decline at 10 μM. Neither steroid was applied at concentrations >10 μM due to limited solubility in Ringer's solution.

Partial agonism by Co 2–1970 was further investigated by measuring effects of 1 and 10 μM Co 2–1970 on the concentration-effect curve for 3α,5α-P (Fig. 7, A and B). At both concentrations, Co 2–1970 reduced levels of potentiation in-

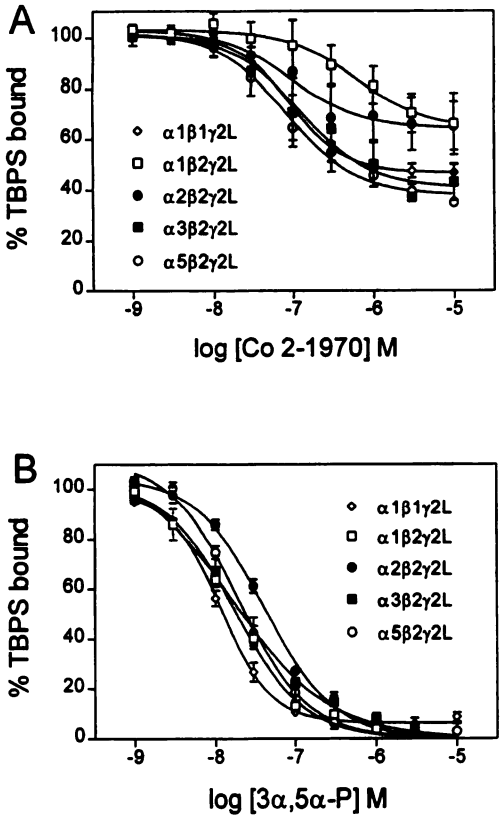


Fig. 5. Co 2–1970 is a limited efficacy and 3α,5α-P is a full efficacy inhibitor of [^{35}S]TBPS binding in stable cell lines expressing recombinant $GABA_A$ receptors. A, Co 2–1970 inhibition of [^{35}S]TBPS binding to α1β1γ2L, α1β2γ2L, α2β2γ2L, α3β2γ2L, and α5β2γ2L receptor complexes. B, 3α,5α-P inhibition of [^{35}S]TBPS binding to α1β1γ2L, α1β2γ2L, α2β2γ2L, α3β2γ2L, and α5β2γ2L receptor complexes. See Table 2 for IC_{50}, I_{max}, and Hill values. The GABA concentration was 5 μM for α1β1γ2L, α2β2γ2L, and α5β2γ2L and 10 μM for α1β2γ2L and α3β2γ2L. These GABA concentrations were found to be the peaks of the biphasic curves for GABA allosteric modulation of TBPS binding determined in separate experiments (data not shown). With the peak GABA concentration for the α1β1γ2L, α1β2γ2L, α2β2γ2L, α3β2γ2L, and α5β2γ2L cell lines, preliminary K_D values were 62 ± 5, 53, 70, 68, and 34 nM, and the corresponding B_{max} values were 9 ± 1, 3.5, 2.0, 2.7, and 1.0 pmol/mg protein, respectively (one experiment except for α1β1γ2L, which involved three experiments) (data not shown).

TABLE 2

Potencies and efficacies of Co 2–1970 and 3α,5α-P for inhibiting TBPS binding to recombinant receptors

Subunit combination	Neuroactive steroid	IC_{50}	I_{max}
		nM	%
α1β1γ2L	Co 2–1970	98 ± 10	54 ± 1
α1β2γ2L		1059 ± 492	43 ± 9
α2β2γ2L		170 ± 91	39 ± 12
α3β2γ2L		254 ± 194	65.1 ± 0.04
α5β2γ2L		93 ± 22	64 ± 2
α1β1γ2L	3α,5α-P	12 ± 2	92.1 ± 0.3
α1β2γ2L		17.5 ± 0.3	103 ± 4
α2β2γ2L		41 ± 3	105 ± 3
α3β2γ2L		20 ± 7	109 ± 3
α5β2γ2L		21 ± 5	112 ± 3

Hill values were 1.0 except for Co 2–1970 in the α1β1γ2L combination (n_H = 1.36 ± 0.20) and 3α,5α-P in the α3β2γ2L (n_H = 0.75 ± 0.02) combinations. Values are mean ± standard error of three independent experiments. See Fig. 5 for the GABA concentration used for each subunit combination.

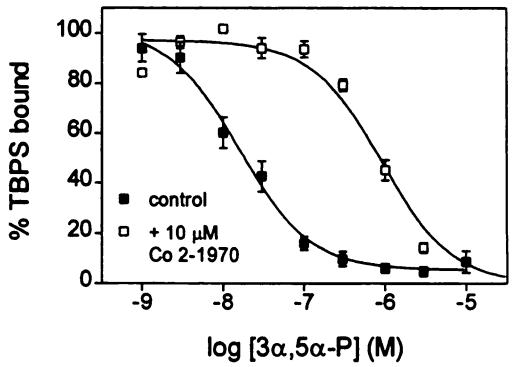


Fig. 6. Co 2–1970 antagonizes the inhibition of [^{35}S]TBPS binding by the full inhibitor 3α,5α-P in cell lines expressing α1β1γ2L receptor complexes. 3α,5α-P inhibition of [^{35}S]TBPS binding in the absence (IC_{50}, 16 ± 2 nM; I_{max}, 97 ± 4%; n_H = 1.0) and presence of 10 μM Co 2–1970 (IC_{50}, 912 ± 44 nM; I_{max}, 97 ± 3%; n_H = 1.0), indicating a decrease in the apparent potency of 3α,5α-P. GABA concentration was 5 μM. Values are mean ± standard error of three independent experiments.

duced by the full efficacy steroid. Co 2–1970 also reduced the small currents activated directly by 3α,5α-P (e.g., Fig. 7A). The potency of this effect seemed to be similar to that for inhibition of the potentiation of GABA responses but was not studied in detail. Inhibitory effects of 1 μM Co 2–1970 on modulation of GABA responses induced by 100 nM 3α,5α-P were not appreciably altered by changing vehicle concentrations over the range of 0.01–1%. This indicated that coprecipitation of steroids was not a major factor in causing the reduction in potentiation. The concentration-effect curves for 3α,5α-P plus Co 2–1970 were rescaled by subtracting potentiation induced by Co 2–1970 alone and calculating fractional responses with respect to control 3α,5α-P. This conversion demonstrated that inhibition was associated with approximately parallel rightward displacement of the 3α,5α-P curve (Fig. 7C). The apparent potency of 3α,5α-P was reduced 3.3-fold by 1 μM Co 2–1970 and 11-fold by 10 μM Co 2–1970 (Table 1). Inhibition was not fully surmountable with the use of 10 μM Co 2–1970. To investigate this effect, the steroids were assayed on maximum GABA responses. The response to 10 mM GABA was slightly reduced by 10 μM Co 2–1970 (fractional response, 0.91 ± 0.031; three experiments). Inhibition became more pronounced in 10 μM Co 2–1970 plus 10 μM 3α,5α-P (fractional response, 0.79 ± 0.062; four experiments). These results suggest that the nonsurmountable component in the inhibition of 3α,5α-P modulation by Co 2–1970 was mainly due to the additional inhibitory effects that micromolar concentrations of steroids typically have on GABA responses. As reported previously (28), inhibition of the maximum GABA response by steroids was associated with an increase in response decay rate.

Discussion

As part of a synthetic program aimed at optimizing the pharmacological properties of neuroactive steroids, several compounds were identified that displayed limited efficacy as allosteric inhibitors of [^{35}S]TBPS binding to the chloride channel-associated site on the $GABA_A$ receptor (24). Indeed, several neuroactive steroids have previously been reported to be limited efficacy inhibitors, including both the endogenous steroid hormone metabolites 5α-pregnan-3α,20α-diol (22)

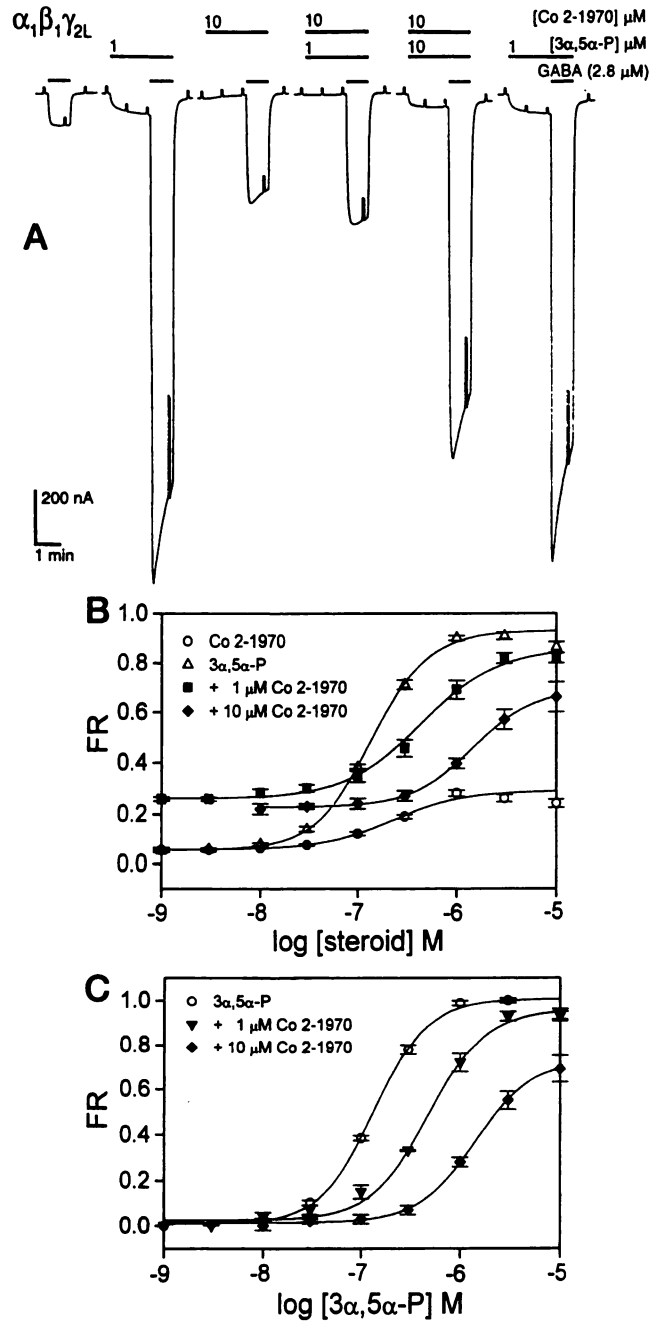


Fig. 7. Functional modulation of GABA-activated membrane current responses in oocytes expressing $\alpha1\beta1\gamma2L$ subunit combinations. A, Sample recordings, taken in sequence from a single oocyte, illustrating high efficacy modulation by 3α,5α-P, low efficacy modulation by Co 2–1970, and surmountable inhibition of 3α,5α-P modulation by Co 2–1970. Although previously exposed to 1 μM 3α,5α-P, the level of modulation induced by 10 μM Co 2–1970 was similar to those measured in concentration-modulation experiments. Holding potential was −70 mV, pulsed to −60 mV (upward deflections) to time drug applications and monitor membrane conductance. *Bars*, drug applications; *downward deflection*, inward current. Drug applications were separated by a 5–10-min wash. B, Concentration-effect curves for potentiation of 5% GABA responses by 3α,5α-P, Co 2–1970, and 3α,5α-P plus two concentrations of Co 2–1970. Optimum EC_{50} and slope values for 3α,5α-P and Co 2–1970 were 140 ± 4 nM and 1.5 ± 0.1 and 220 ± 20 nM and 1.2 ± 0.1, respectively. Optimum EC_{50} and slope values for 3α,5α-P plus 1 μM Co 2–1970 and 3α,5α-P plus 10 μM Co 2–1970 were 460 ± 60 nM and 1.1 ± 0.1 and 1500 ± 500 nM and 1.4 ± 0.4, respectively. C, Same data scaled and replotted to illustrate the rightward displacement of the 3α,5α-P curve induced by Co 2–1970.

and 5β-tetrahydrodeoxycorticosterone (18), as well as several synthetic neuroactive steroids (23, 24). Of the synthetic neuroactive steroids, the limited efficacy inhibitor Co 2–1970 was selected for further characterization.

In rat brain cortical membranes, Co 2–1970 produces a concentration-dependent rightward shift in the 3α,5α-P concentration-effect curve for inhibition of [^{35}S]TBPS binding. Co 2–1970 also decreases the apparent efficacy of the full inhibitor 3α,5α-P in a concentration-dependent manner. The limited efficacy and antagonistic properties of Co 2–1970 observed with [^{35}S]TBPS suggest that it is a partial agonist for the neuroactive steroid binding site. If Co 2–1970 is a true partial agonist for the neuroactive steroid site, these properties should be reflected with radioligands for other sites on the receptor coupled to the neuroactive steroid site. Consistent with the [^{35}S]TBPS results, Co 2–1970 enhances the binding of the benzodiazepine site ligand [^{3}H]flunitrazepam with limited efficacy and also decreases the apparent potency of 3α,5α-P for enhancement of [^{3}H]flunitrazepam binding.

In contrast to the limited efficacy modulation of [^{3}H]flunitrazepam and [^{35}S]TBPS binding, Co 2–1970 is virtually inactive as a modulator of [^{3}H]muscimol binding to the GABA recognition site. By analogy to the lack of effect of Co 2–1970 on [^{35}S]TBPS binding in the absence of added GABA, the inactivity of Co 2–1970 in the [^{3}H]muscimol assay may be related to the removal of endogenous GABA in the membrane preparation steps required to obtain a high level of [^{3}H]muscimol binding. Thus, GABA seems to be required for Co 2–1970 to either bind to the neuroactive steroid site or elicit a conformational change in the receptor complex resulting in modulation of ligand binding to coupled sites. The latter possibility is favored because Co 2–1970 reduces the apparent potency of 3α,5α-P for enhancement of [^{3}H]muscimol, indicating that it has affinity for the neuroactive steroid site under these conditions. Thus, Co 2–1970 has a pure antagonist rather than a partial agonist profile with respect to [^{3}H]muscimol binding, probably due to removal of endogenous GABA.

In rat brain cortical membranes, Co 2–1970 modulates [^{35}S]TBPS and [^{3}H]flunitrazepam binding with a profile consistent with partial agonism. These results may be complicated, however, by the expression in cortex of a diversity of subunits that combine to form pharmacologically heterogeneous $GABA_A$ receptor populations (2). Indeed, the limited efficacy and the rightward shift in the 3α,5α-P concentration-effect curve for inhibition of [^{35}S]TBPS binding observed for 5α-pregnan-3α,20α-diol have been interpreted as evidence for neuroactive steroid binding site heterogeneity (17). To directly address the question of subtype selectivity, Co 2–1970 was examined in five stable cell lines expressing recombinant receptor combinations thought to be among the most prevalent in the brain. In all cases, Co 2–1970 was found to be a limited efficacy inhibitor of [^{35}S]TBPS binding. Although differences in potency were observed, the limited efficacy in rat cortex does not seem to be due to inactivity of Co 2–1970 in some prevalent receptor subtype. Moreover, Co 2–1970 antagonizes the 3α,5α-P-induced inhibition of [^{35}S]TBPS binding in membranes from cells expressing $\alpha1\beta1\gamma2L$ receptor complexes. Thus, Co 2–1970 exhibits a profile in recombinant receptors that is consistent with partial agonism and not with subtype selectivity.

Electrophysiological studies in oocytes expressing $\alpha1\beta1\gamma2L$ subunit combinations show that Co 2–1970 is a

limited efficacy potentiator of GABA-activated currents. Moreover, coapplication of Co 2–1970 with the full efficacy steroid 3α,5α-P results in reductions in levels of potentiation. This inhibition is surmountable and is associated with a decrease in the apparent potency for 3α,5α-P. As described for the allosteric binding studies, these results are consistent with Co 2–1970 acting as a partial agonist competing for the same modulatory site on the $GABA_A$ receptor complex recognized by 3α,5α-P.

In the oocyte assays, maximum potentiation induced by Co 2–1970 involved a 6-fold increase in the 5% control GABA current compared with an 18-fold increase for the full efficacy modulator 3α,5α-P. The level of modulation by Co 2–1970 is greater than the potentiation induced by full efficacy benzodiazepines, which typically increase responses by only ~1-fold[2] (also see Ref. 9), but is distinctly lower than that induced by the barbiturates such as pentobarbital, which, like 3α,5α-P, increase responses by ~18-fold.[2] At the single-channel level, benzodiazepines modulate $GABA_A$ receptors by increasing the frequency of channel opening, an effect usually ascribed to an increase in agonist affinity (42). In contrast, barbiturates modulate $GABA_A$ receptors by increasing the proportion of openings with long durations, an effect on channel gating (43). Steroids such as 3α,5α-P potentiate GABA responses by increasing the frequency of channel opening and by prolonging open durations (44). For low efficacy steroids such as Co 2–1970, it will be interesting to determine whether the decrease in modulation relative to full efficacy steroids is selectively due to a reduction in effects on channel opening frequency or duration or whether effects on both parameters are reduced in parallel.

In both binding and electrophysiological assays, the potency of Co 2–1970 modulation given by its IC_{50} or EC_{50} value is generally similar to its antagonist dissociation constant (K_i or K_b value) calculated from the decrease in apparent potency of 3α,5α-P in the presence of Co 2–1970 with the Gaddum-Schild relationship. The similarity of IC_{50} or EC_{50} and K_i or K_b values suggests that the modulation and antagonism displayed by Co 2–1970 are mediated by a common mechanism. In general, the differences between the IC_{50} or EC_{50} values and the K_i or K_b values were ≤2-fold, although at the high concentration of Co 2–1970 in the TBPS rat cortex and electrophysiology α1β1γ2L assays, the discrepancy was greater. In these cases, the potency of Co 2–1970 as a modulator was higher than its potency as an antagonist. The lower-than-expected antagonist potency may be due to complications arising from the agonistic activity of Co 2–1970 because the Gaddum-Schild relationship was derived to describe the action of pure antagonists. A better estimate of the dissociation constant awaits a direct binding assay for neuroactive steroids.

Taken together, the limited efficacy and antagonist activity of Co 2–1970 observed in allosteric binding assays in native and recombinant $GABA_A$ receptors and confirmed electrophysiologically in recombinant receptors provide compelling evidence for partial agonism of Co 2–1970 at the neuroactive steroid site. Partial agonism has also been observed for the presumed desoxycorticosterone metabolite 5β-tetrahydrodeoxycorticosterone.[3] Future studies will address the *in vivo* pharmacological profile of Co 2–1970 in terms of both its side effect profile and its potential to antagonize the behavioral effects of full agonist neuroactive steroids.

Acknowledgments

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