

# Behavioral Effects of $\gamma$ -Hydroxybutyrate, Its Precursor $\gamma$ -Butyrolactone, and GABA<sub>B</sub> Receptor Agonists: Time Course and Differential Antagonism by the GABA<sub>B</sub> Receptor Antagonist 3-Aminopropyl(diethoxymethyl)phosphinic Acid (CGP35348)

Wouter Koek, Susan L. Mercer, Andrew Coop, and Charles P. France

*Departments of Psychiatry and Pharmacology, the University of Texas Health Science Center at San Antonio, San Antonio, Texas (W.K., C.P.F.); Department of Pharmaceutical Sciences, Lipscomb University College of Pharmacy, Nashville, Tennessee (S.L.M.); and Department of Pharmaceutical Sciences, University of Maryland, Baltimore, Maryland (A.C.)*

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## ABSTRACT

$\gamma$ -Hydroxybutyrate (GHB) is used therapeutically and recreationally. The mechanism by which GHB produces its therapeutic and recreational effects is not entirely clear, although GABA<sub>B</sub> receptors seem to play an important role. This role could be complex, because there are indications that different GABA<sub>B</sub> receptor mechanisms mediate the effects of GHB and the prototypical GABA<sub>B</sub> receptor agonist baclofen. To further explore possible differences in underlying GABA<sub>B</sub> receptor mechanisms, the present study examined the effects of GHB and baclofen on operant responding and their antagonism by the GABA<sub>B</sub> receptor antagonist 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP35348). Pigeons were trained to peck a key for access to food during response periods that started at different times after the beginning of the session. In these pigeons, GHB, its precursor  $\gamma$ -butyrolactone (GBL), and the

GABA<sub>B</sub> receptor agonists baclofen and 3-aminopropyl(methyl)phosphinic acid hydrochloride (SKF97541) decreased the rate of responding in a dose- and time-dependent manner. CGP35348 shifted the dose-response curve of each agonist to the right, but the magnitude of the shift differed among the agonists. Schild analysis yielded a  $pA_2$  value of CGP35348 to antagonize GHB and GBL [i.e., 3.9 (3.7–4.2)] that was different ( $P = 0.0011$ ) from the  $pA_2$  value to antagonize baclofen and SKF97541 [i.e., 4.5 (4.4–4.7)]. This finding is further evidence that the GABA<sub>B</sub> receptor mechanisms mediating the effects of GHB and prototypical GABA<sub>B</sub> receptor agonists are not identical. A better understanding of the similarities and differences between these mechanisms, and their involvement in the therapeutic effects of GHB and baclofen, could lead to more effective medications with fewer adverse effects.

$\gamma$ -Hydroxybutyric acid (GHB) is an endogenous molecule, a marketed therapeutic drug, and a drug of abuse. GHB is a putative neuromodulator (Maitre, 1997) involved in the regulation of sleep and used clinically to treat narcolepsy (Fuller and Hornfeldt, 2003) and alcoholism (Poldrugo and Adolorato, 1999). GHB is also used recreationally (Gonzalez and Nutt, 2005). The precise mechanism by which GHB exerts its various effects is unknown.

GHB binds to specific sites in brain (Benavides et al., 1982) and to GABA<sub>B</sub> receptors (Mathivet et al., 1997). At present,

there is little evidence that specific GHB binding sites mediate the in vivo effects of GHB (Wong et al., 2004). Instead, many studies suggest that GABA<sub>B</sub> receptors are particularly important for various behavioral effects of GHB, including hypolocomotion (Kaupmann et al., 2003), catalepsy (Carter et al., 2005), ataxia (Goodwin et al., 2005), loss of righting (Carai et al., 2001), decreased operant responding (Goodwin et al., 2005), and discriminative stimulus effects (Winter, 1981; Colombo et al., 1998; Carter et al., 2003, 2009; Koek et al., 2004, 2006). All of these effects of GHB are also produced by the prototypical GABA<sub>B</sub> receptor agonist baclofen (Carter et al., 2003, 2004, 2005), consistent with the involvement of GABA<sub>B</sub> receptors in the effects of GHB.

Although GABA<sub>B</sub> receptors probably mediate behavioral

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**ABBREVIATIONS:** GHB,  $\gamma$ -hydroxybutyrate; GBL,  $\gamma$ -butyrolactone; SKF97541, 3-aminopropyl(methyl)phosphinic acid hydrochloride; CGP35348, 3-aminopropyl(diethoxymethyl)phosphinic acid; CGP62349, [3-[1-(*R*)-[[[2S]-2-hydroxy-3-[hydroxyl[4-methoxyphenyl)methyl]phosphiny]propyl]-amino]ethyl]-benzoic acid; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; MK-801, dizocilpine.

effects that GHB has in common with baclofen, there is growing evidence that the underlying GABA<sub>B</sub> receptor mechanisms are not identical. One line of evidence is from studies that examined the interactions of GHB and baclofen with antagonists at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors. The NMDA antagonist dizocilpine (MK-801) enhances GHB-induced catalepsy in rodents (Sevak et al., 2004, 2005; Koek and France, 2008). The cataleptic effects of GHB are enhanced not only by MK-801 but also by other drugs with NMDA antagonist activity, such as phencyclidine (PCP) and ketamine. However, these NMDA antagonists do not affect the cataleptic effects of baclofen (Koek and France, 2008). Similar interactions have been observed in drug discrimination studies; PCP enhances the discriminative stimulus effects of GHB but not of baclofen (Koek et al., 2007a). Differential enhancement of the effects of GHB and baclofen by NMDA antagonists suggests that the GABA<sub>B</sub> receptor mechanisms involved in the effects of GHB and baclofen may not be identical.

A second line of evidence that the GABA<sub>B</sub> receptor mechanisms underlying the effects of GHB and baclofen may be different is from antagonism studies. The GABA<sub>B</sub> receptor antagonist CGP35348 antagonizes the discriminative stimulus effects of GHB and baclofen, consistent with the involvement of GABA<sub>B</sub> receptors, but is less potent in antagonizing these effects of GHB than those of baclofen (Carter et al., 2006). Recently, we reported that CGP35348 was also less potent in antagonizing the cataleptic effects of GHB than those of baclofen (Koek et al., 2007b). Together, these findings suggest a possible role for GABA<sub>B</sub> receptor subtypes or different interactions with the same GABA<sub>B</sub> receptor in the behavioral effects of GHB and baclofen.

A detailed characterization of antagonist actions requires complete dose-response curves of the agonist in the presence of several doses of the antagonist. Such data are often analyzed by Schild regression, which yields information about the nature of the antagonism and the potency of the antagonist. This method compares the pattern of antagonism to that predicted by the simple competitive model (i.e., the agonist and antagonist compete for the same recognition sites on the receptor). If the Schild regression has a slope of unity, this is consistent with simple competitive antagonism; deviations from unity can signify noncompetitive antagonism, nonequilibrium steady states, or receptor-population heterogeneity (Kenakin, 1997). A limitation of the studies examining antagonism of GHB and baclofen that we have conducted to date is that their results could not be analyzed by Schild regression. The present study, aimed at remedying this limitation, is an effort to characterize in detail the antagonism by CGP35348 of behavioral effects of GHB, its precursor  $\gamma$ -butyrolactone (GBL), and the GABA<sub>B</sub> receptor agonists baclofen and SKF97541. The behavioral measure used was decreased operant responding, assessed in a procedure that provided information about the time course of agonist and antagonist effects. The results confirm preliminary findings of differential antagonism by CGP35348 of GHB and baclofen, and suggest that the underlying GABA<sub>B</sub> receptor mechanisms are different, which may have implications for their different profiles of preclinical and clinical activities.

## Materials and Methods

**Animals.** Ten adult white Carneau pigeons (*Columbia livia*; Palmetto Pigeon Plant, Sumter, SC) were individually housed under a 12:12-h light/dark cycle. They had free access to water and were maintained between 80 and 90% of their free-feeding weight by food (Purina Pigeon Checkers, St. Louis, MO) received during experimental sessions and supplemental postsession feedings (Purina Pigeon Checkers or mixed grain). All subjects had drug discrimination histories (Koek et al., 2006) and had not received any drug for at least one month before the start of the current study. Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee (The University of Texas Health Science Center at San Antonio) and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

**Apparatus.** Experiments were conducted in sound attenuating, ventilated chambers (BRS/LVE, Laurel, MD) equipped with a response key that could be illuminated by a red light. After completion of each fixed ratio, the key light was extinguished for 4 s, during which time a white light illuminated the hopper where food (Purina Pigeon Checkers) was available. Chambers were connected by an interface (MED Associates Inc., St. Albans, VT) to a computer that used MED-PC IV software (MED Associates Inc.) to monitor and control inputs and outputs and to record the data.

**Procedure.** The procedure was similar to that described in detail by Schlinger and Poling (1988). In brief, pigeons trained to peck the response key for access to food were exposed to 12-h overnight sessions that were conducted four times per week, Monday through Thursday. During each session, the key was illuminated during 10 response periods, each starting at a different time after the beginning of the session (i.e., 0, 15, 30, and 60 min and 2, 4, 6, 8, 10, and 12 h). When the key was illuminated, 20 responses resulted in 4-s access to food (i.e., fixed ratio 20). A response period ended after five food presentations or 5 min, whichever occurred first. Between response periods the key light was off, and responses had no programmed consequence. Response periods began with a brief (0.25 s) operation and illumination of the hopper (i.e., a brief auditory and visual stimulus).

Once responding stabilized under the fixed-ratio schedule during each of the 10 response periods (i.e., no visible trend was evident for at least five consecutive sessions), subjects received an intramuscular injection of physiological saline before each session. Monday and Wednesday sessions were always preceded by a saline injection. If responding during a saline session did not differ by more than 20% from responding during the previous saline session, an antagonist and/or agonist was given before the next session (i.e., on Tuesday or Thursday). Otherwise, a saline session was conducted. Agonists were given immediately before the session, and the antagonist was given 10 min before an agonist.

**Data Analysis.** Response rates were calculated for each response period by dividing the number of responses by the duration (in seconds) of the period. For each animal, response rates during the periods that started at different times after drug administration were expressed as a percentage of the corresponding control values obtained during the previous vehicle session. The response rates during drug test sessions, expressed as percentage control, were averaged across animals, and mean values  $\pm$  S.E.M. were plotted as a function of dose and time after drug administration.

To calculate doses needed to produce 50% of the maximal response (ED<sub>50</sub>) and their 95% confidence limits, the linear portion of the dose-response curves was analyzed by log-linear regression (Tallarida, 2000) of data from individual subjects by use of GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com), with the following equation: effect = slope  $\times$  log(dose) + intercept. The linear portion comprised the data points at doses immediately below and above 50% and included not more than one dose with an effect larger than 80% and not more than one dose

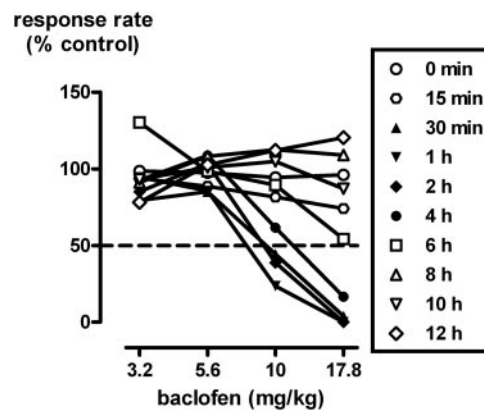
with an effect smaller than 20%. For each dose-response curve fitted to more than two doses, the replicates test (also called the lack-of-fit test; Draper and Smith, 1998) implemented in GraphPad Prism was used to examine whether the log dose-response data used in the log-linear regression deviated from linearity. *F* ratio tests in GraphPad Prism were used to compare dose-response curves with respect to their slopes and intercepts. For example, a nonsignificant *F* ratio for slopes and a significant *F* ratio for intercepts show that dose-response curves are parallel but occupy different positions on the dose axis.

To examine the onset and duration of agonist effects, ED<sub>50</sub> values for each agonist were plotted as a function of time after drug administration. Differences among ED<sub>50</sub> values were analyzed by use of the *F* ratio test implemented in GraphPad Prism and with the common slope calculated by GraphPad Prism to constrain the fit of the parallel-line assay (Tallarida, 2000), as detailed elsewhere (Koek et al., 2006). Dose-response data obtained at the time that the ED<sub>50</sub> values were lowest (i.e., when the agonist seemed to reach peak effect) were used to examine antagonist effects. For each agonist, differences among its dose-response curves to decrease response rate in the presence of different doses of antagonist were analyzed by simultaneously fitting straight lines to the linear portion of the dose-response curves. Differences among the slopes and intercepts of the curves were analyzed with the *F* ratio test, and ED<sub>50</sub> values and potency ratios were calculated by parallel-line analysis (Tallarida, 2000). For each agonist, potency ratios were used to calculate an apparent pA<sub>2</sub> value for the antagonist according to the methods described by Arunlakshana and Schild (1959). The Schild plots were analyzed with the *F* ratio test to examine whether the slopes of the Schild regressions deviated significantly from -1, and then to examine differences among pA<sub>2</sub> values by comparing the following models of increasing complexity (i.e., increasing number of parameters): 1) the same pA<sub>2</sub> value for all agonists; 2) the same pA<sub>2</sub> value for GHB and SKF97541, the same pA<sub>2</sub> value for GHB and GBL; and 3) an individual pA<sub>2</sub> value for each agonist. Thus, Schild plots were used to examine whether the effects of the antagonist differed among agonists. In addition, Schild analyses of data obtained at various times after the administration of the antagonist were used to examine the duration of antagonist activity.

**Drugs.** GHB, GBL, and (±)-baclofen were purchased from Sigma-Aldrich (St. Louis, MO). CGP35348 (sodium salt) and SKF97541 hydrochloride were synthesized at the University of Maryland. All compounds were dissolved in physiological saline (0.9% NaCl), with the exception of GHB, which was dissolved in sterile water. All compounds were injected intramuscularly in a volume of 0.1 to 1.0 ml. Doses are expressed as the form of the drug listed above.

## Results

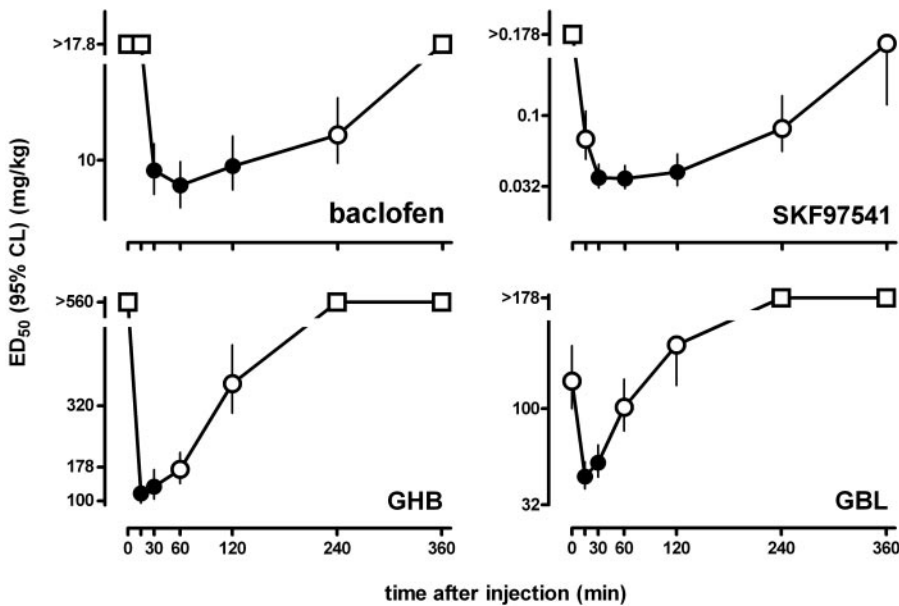
Under control conditions, key peck responses occurred at a rate that varied not more than 20% within pigeons, and that varied from 0.83 to 1.94 responses per second between pigeons. Baclofen decreased the rate of responding in a dose- and time-dependent manner (Fig. 1). Responding was decreased to less than 50% of control between 30 min and 4 h after the injection of baclofen (Fig. 1, ▲, ▼, ◆, and ●), but not at shorter or longer intervals (Fig. 1, ○, □, △, ▽, and ◇). The dose-response data obtained at 30 min and 1, 2, and 4 h after 5.6, 10, and 17.8 mg/kg baclofen were analyzed by log-linear regression. The four dose-response curves, which did not deviate from linearity (replicates test, *P* > 0.20), had a common slope [*F*(3,84) = 0.31, *P* = 0.82] but not a common ED<sub>50</sub> [*F*(3,87) = 2.73, *P* = 0.049]. The ED<sub>50</sub> of baclofen to decrease responding was lowest at 60 min after injection [i.e., 8.6 (95% confidence limits: 7.1–10) mg/kg; Fig. 2; Table 1] and was not different at 30 and 120 min [*F*(1,43) ≤ 1.69, *P* ≥ 0.20] but was



**Fig. 1.** Effects of baclofen, administered intramuscularly immediately before the session, on fixed ratio 20 responding in a one-key, food-reinforced procedure (*n* = 5–9). Each 12-h session consisted of 10 5-min response periods starting at different times after the beginning of the session. For each of these response periods, the rate of key peck responding is plotted as a function of dose. Symbols represent mean ± S.E.M.; S.E.M. values are contained by the symbol.

higher at 240 min [*F*(1,43) = 8.28, *P* = 0.0062]. Based on these results, baclofen seemed to reach peak effect 30 to 120 min after injection. The other drugs reached peak effect at 30 to 120 (SKF97541) or at 15 to 30 min (GHB, GBL). Thus, at 30 min after injection, all drugs were maximally active.

When injected alone at a dose of 32 mg/kg, the GABA<sub>B</sub> receptor antagonist CGP35348 did not alter the rate of responding (data not shown). The response rate, expressed as a percentage of saline control, did not significantly change during the session and varied between 98 and 109% (S.E.M., 2.5–6.3). When injected 10 min before each agonist, CGP35348 dose-dependently shifted the dose-response curve of each agonist to the right (Fig. 3, top and middle; Table 1). However, the extent and nature of these shifts seemed to differ among some of the agonists. To examine these apparent shifts, the linear portion of each dose-response curve (see *Materials and Methods*) was analyzed by log-linear regression. None of the dose-response data used in the regression analyses deviated from linearity [replicates test: *P* values ranged from a minimum of 0.13 obtained for *F*(1,9) = 2.73 to a maximum of 0.98 for *F*(2,14) = 0.02]. CGP35348 shifted the dose-response curves of baclofen and SKF97541 to the right [common intercept: *F*(3,49) ≥ 7.80, *P* ≤ 0.0002], in a parallel manner [common slope: *F*(3,46) ≤ 1.29, *P* ≥ 0.29], and to an apparently similar extent. At the same doses, CGP35348 shifted the dose-response curves of GHB [common intercept: *F*(3,41) = 7.53, *P* = 0.0004] and GBL [common intercept: *F*(3,47) = 3.37, *P* = 0.026], but apparently less extensively, and in the case of GHB, in a nonparallel manner [GHB, common slope: *F*(3,41) = 6.34, *P* = 0.0012; GBL, common slope: *F*(3,44) = 2.02, *P* = 0.12]. The antagonist effects of CGP35348 were quantified by means of Schild regression plots (Fig. 3, bottom). These plots, with a common slope [*F*(3,4) = 0.47, *P* = 0.72] not different from -1 [*F*(1,10) = 2.04, *P* = 0.18], yielded the following pA<sub>2</sub> values (Table 1): 4.46 (95% confidence limits: 4.10–4.82) for baclofen, 4.63 (4.17–5.09) for SKF97541, 3.97 (3.47–4.47) for GHB, and 3.91 (2.98–4.85) for GBL. The confidence interval of the estimated pA<sub>2</sub> value was wider for GBL (i.e., 1.87) than for baclofen, SKF97541, and GHB (i.e., 0.72, 0.92, and 1, respectively), indicating that the Schild regression fitted the data



**Fig. 2.** Effects of baclofen, SKF97541, GHB, and GBL, administered intramuscularly immediately before the session, on fixed ratio 20 responding in a one-key, food-reinforced procedure in pigeons ( $n = 4-9$ ). The potency to decrease the rate of key peck responding is plotted as a function of time from the beginning of the session. Circles represent  $ED_{50}$  and 95% confidence limits. For each drug, open circles indicate  $ED_{50}$  values that differed significantly from the lowest observed value, and closed circles indicate  $ED_{50}$  values that did not. Squares represent interval estimates of  $ED_{50}$  values.

TABLE 1

$ED_{50}$  values (in milligrams per kilogram) for effects of agonists on food-reinforced responding in pigeons at different injection-test intervals and  $pA_2$  values of CGP35348 [in  $-\log(\text{mol/kg})$ ] to antagonize these effects. Numbers in parentheses are the 95% confidence limits.

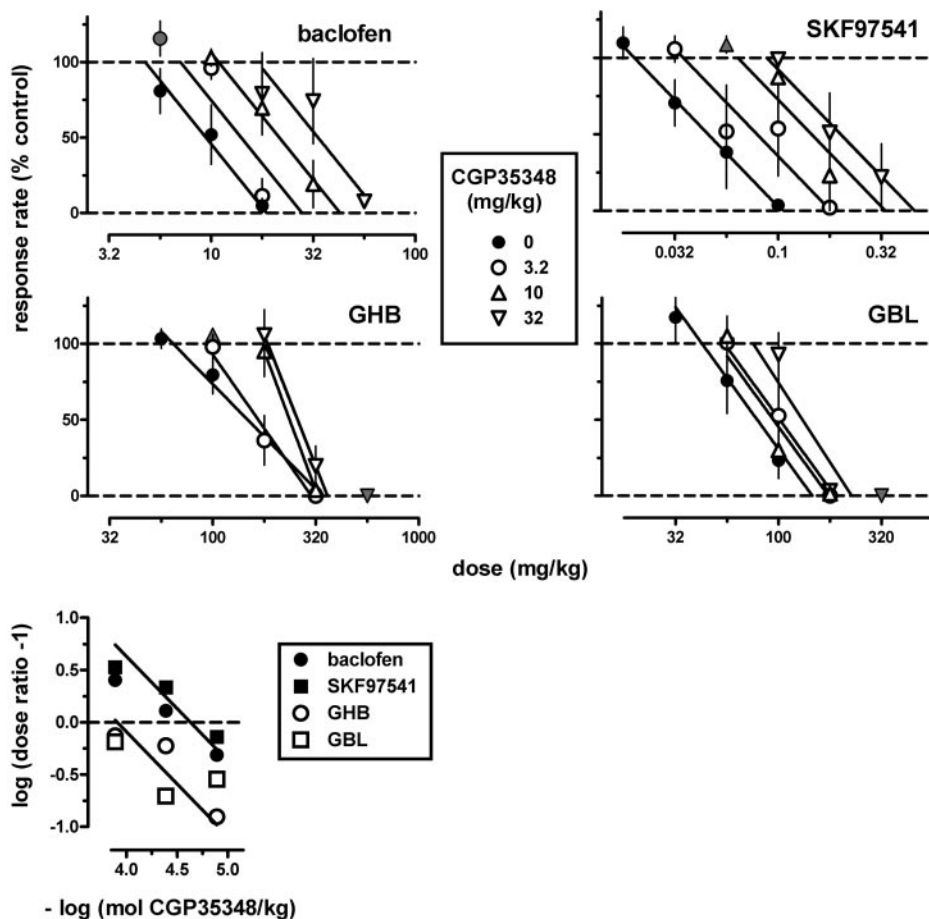
Agonist	Time	CGP35348				$pA_2$
		0	3.2	10	32	
	<i>min</i>		<i>mg/kg</i>			
Baclofen	30	9.4 (7.4-12)	14 (10-N.D.)	22 (16-30)	33 (24-47)	4.46 (4.10-4.82)
	60	8.6 (7.1-10)	15 (12-N.D.)	18 (14-22)	31 (25-40)	4.49 (3.93-5.05)
	120	9.1 (7.9-10)	14 (12-17)	19 (16-22)	34 (28-40)	4.46 (4.07-4.85)
	240	12 (9.6-15)	17 (13-N.D.)	22 (17-28)	36 (27-48)	4.34 (3.95-4.73)
SKF97541	15	0.077 (0.047-0.14)	0.12 (0.068-N.D.)	0.19 (N.D.)	0.42 (0.20-N.D.)	4.46 (4.11-4.81)
	30	0.046 (0.034-0.063)	0.079 (0.057-0.11)	0.15 (N.D.)	0.20 (0.14-0.30)	4.63 (4.17-5.09)
	60	0.046 (0.036-0.059)	0.078 (0.061-0.098)	0.11 (0.085-0.15)	0.18 (0.14-0.24)	4.54 (4.09-4.98)
	120	0.051 (0.041-0.064)	0.079 (0.064-0.098)	0.11 (0.085-0.14)	0.22 (N.D.-30)	4.52 (4.21-4.82)
	240	0.091 (0.067-0.13)	0.10 (0.074-0.16)	0.15 (0.10-N.D.)	0.30 (0.18-N.D.)	4.18 (3.92-4.43)
GHB	15	150 (120-190)	200 (160-250)	250 (190-N.D.)	250 (190-N.D.)	4.14 (3.26-5.02)
	30	150 (130-180)	170 (140-200)	240 (210-270)	260 (220-N.D.)	3.97 (3.47-4.47)
	60	160 (130-200)	180 (150-210)	280 (230-N.D.)	340 (280-400)	3.96 (2.38-5.54)
GBL	30	79 (67-93)	100 (82-130)	94 (76-120)	130 (N.D.-170)	3.91 (2.98-4.85)
	60	100 (81-130)	110 (86-140)	140 (130-150)	150 (120-N.D.)	3.72 (3.24-4.19)

N.D., not determined because the confidence limit could not be calculated.

obtained with GBL less well than those obtained with the other drugs. To examine similarities and differences among the  $pA_2$  values obtained for CGP35348 with each of the four agonists, the following models of increasing complexity (i.e., larger number of parameters) were compared by means of  $F$  tests: model 1) a common  $pA_2$  value for all agonists; model 2) a common  $pA_2$  value for baclofen and SKF97541 and a common  $pA_2$  value for GHB and GBL; and model 3) an individual  $pA_2$  value for each agonist. Model 2 fitted the data better than model 1 did [ $F(1,10) = 20.74$ ,  $P = 0.0011$ ], which indicates that the data could not be adequately fitted with a single  $pA_2$  value. However, adding more parameters, by assuming that the  $pA_2$  values differed for each agonist, did not further increase the fit [comparison of model 3 with model 2:  $F(2,8) = 0.42$ ,  $P = 0.67$ ]. Thus, model 2 was the simplest model that could be fitted to the Schild regression data obtained with all four drugs and consisted of one plot for baclofen and SKF97541 and one for GHB and GBL. Based on these plots, the  $pA_2$  value of CGP35348 was 4.54 (4.36-4.73) to antagonize baclofen and SKF97541, and was 3.94 (3.66-

4.23) to antagonize GHB and GBL. Thus, CGP35348 was 4-fold less potent to antagonize the response rate decreasing effects of GHB and GBL than to antagonize those of baclofen and SKF97541.

SKF97541 dose-dependently decreased response rate not only at 30 min but also at shorter and longer injection-test intervals (Fig. 4, left, top to bottom, and right, top and middle). None of the dose-response data used in the regression analyses deviated from linearity [replicates test:  $P$  values ranged from a minimum of 0.19 obtained for  $F(2,15) = 1.88$  to a maximum of 0.98 for  $F(2,14) = 0.02$ ]. CGP35348, injected 10 min before SKF97541, dose-dependently shifted the dose-response curves of SKF97541 at each interval to the right [common intercept: 15 min,  $F(3,42) = 3.53$ ,  $P = 0.023$ ; 30 min,  $F(3,49) = 7.80$ ,  $P = 0.0002$ ; 60 min,  $F(3,50) = 13.13$ ,  $P < 0.0001$ ; 120 min,  $F(3,46) = 12.38$ ,  $P < 0.0001$ ; 240 min,  $F(4,29) = 12.38$ ,  $P = 0.009$ ], in a parallel manner [common slope: 15 min,  $F(3,39) = 0.44$ ,  $P = 0.73$ ; 30 min,  $F(3,46) = 0.50$ ,  $P = 0.69$ ; 60 min,  $F(3,47) = 0.99$ ,  $P = 0.41$ ; 120 min,  $F(3,43) = 2.45$ ,  $P = 0.077$ ; 240 min,  $F(3,47) = 1.84$ ,  $P = 0.15$ ]. However,



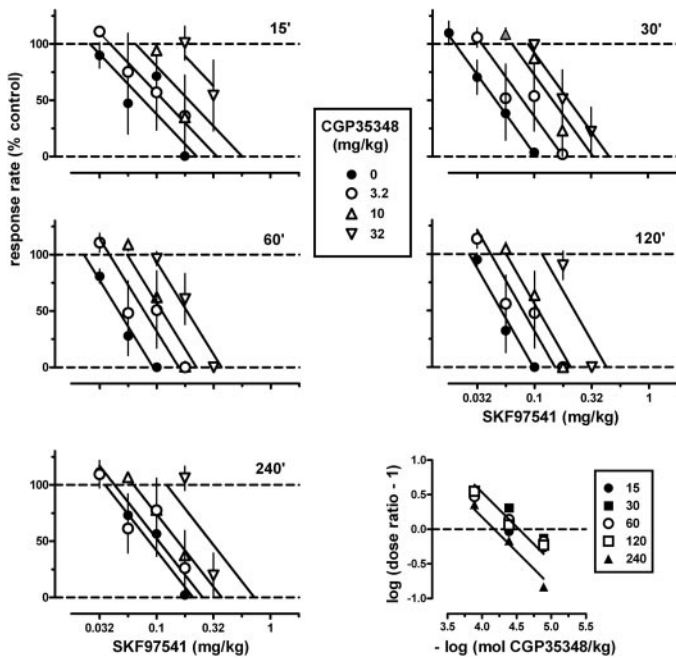
**Fig. 3.** Top and middle, attenuation by CPG35348 of response rate-decreasing effects of baclofen, SKF97541, GHB, and GBL in pigeons ( $n = 5-6$ ). CPG35348 or saline was injected 10 min before each agonist, and 30 min after the agonist was injected, key peck responding was measured. For each agonist, the rate of key peck responding, expressed as a percentage of saline control, is plotted as a function of dose after pretreatment with saline (●) or different doses of CPG35348 (○, △, ▽, data comprising the linear portion of the dose-response curves, and fitted with log-linear regression lines; gray-filled symbols, data obtained with the same doses of CPG35348 as the corresponding open symbols, but not part of the linear portion of the dose-response curves, and not used in the regression calculations (see “Data Analysis”). Symbols represent mean  $\pm$  S.E.M.; if not shown, S.E.M. values are contained by the symbol. Bottom, Schild regression plots for antagonism by CPG35348 of the response rate-decreasing effects of baclofen, SKF97541, GHB, and GBL. Dose ratios are the  $ED_{50}$  values of SKF97541 in the presence of CPG35348 (3.2–32 mg/kg) divided by the  $ED_{50}$  value after pretreatment with saline.  $ED_{50}$  values were calculated from the regression lines shown in the top and middle. Data obtained with each agonist could be fitted with a regression line with a slope of  $-1$ . Calculated from these regression lines, the  $pA_2$  value of CPG35348 ranged from 3.91 (95% confidence limits, 2.98–4.85) for GBL to 4.63 (4.17–5.09) for SKF97541.

the  $pA_2$  values for CPG35348 obtained at these intervals were not the same (Fig. 4, bottom right; Table 1). The Schild regression plots, with a common slope [ $F(4,5) = 3.55$ ,  $P = 0.10$ ] not different from  $-1$  [ $F(1,13) = 2.48$ ,  $P = 0.14$ ], yielded a  $pA_2$  value at 240 min after the injection of SKF9754 [i.e., 4.18 (3.93–4.43)] that differed from the values obtained at shorter intervals (Table 1). To examine similarities and differences among the  $pA_2$  values obtained at each of the intervals, the following models of increasing complexity (i.e., larger number of parameters) were compared by means of  $F$  tests: model 1) a common  $pA_2$  value for all intervals; model 2) a common  $pA_2$  value for the 15- to 120-min intervals, and a  $pA_2$  value for the 240-min interval; and model 3) an individual  $pA_2$  value for each interval. Model 2 fitted the data better than model 1 [ $F(1,13) = 14.87$ ,  $P = 0.002$ ], which indicates that the data could not be adequately fitted with a single  $pA_2$  value. However, adding more parameters, by assuming the  $pA_2$  values to differ for each interval, did not further increase the fit [comparison of model 3 with model 2:  $F(3,10) = 0.62$ ,  $P = 0.62$ ]. Thus, model 2 was the simplest model that could be fitted to the Schild regression data obtained at all five intervals and consisted of one plot for the 15- to 120-min intervals and one plot for the 240-min interval. Based on these plots, the  $pA_2$  value of CPG35348 was 4.53 (4.44–4.63) to antagonize SKF97541 at 15 to 120 min after its injection, and it was 4.18 (3.93–4.43) at 240 min. Thus, the potency of CPG35348 to antagonize SKF97541 was not different from 15 to 120 min after the injection of SKF97541 (i.e., 25–130 min after CPG35348); however, 250 min after the injection of

CPG35348, its potency decreased approximately 2-fold. A similar trend was apparent when CPG35348 was used to antagonize the effects of baclofen at various time intervals (Fig. 5), but this failed to reach statistical significance [ $F(1,10) = 1.46$ ,  $P = 0.25$ ]. The  $pA_2$  values obtained with GHB and GBL did not differ across intervals [GHB:  $F(2,5) = 0.40$ ,  $P = 0.69$ ; GBL:  $F(1,4) = 0.63$ ,  $P = 0.47$ ] but were lower than those obtained with baclofen and SKF97541 at the same interval [30 min: GHB versus baclofen,  $F(1,4) = 11.40$ ,  $P = 0.028$ ; GHB versus SKF97541,  $F(1,4) = 17.39$ ,  $P = 0.014$ ; GBL versus baclofen,  $F(1,4) = 5.51$ ,  $P = 0.079$ ; GBL versus SKF97541,  $F(1,4) = 8.85$ ,  $P = 0.041$ ; 60 min: GHB versus baclofen,  $F(1,3) = 7.63$ ,  $P = 0.07$ ; GHB versus SKF97541,  $F(1,3) = 12.80$ ,  $P = 0.037$ ; GBL versus baclofen,  $F(1,4) = 20.42$ ,  $P = 0.011$ ; GBL versus SKF97541,  $F(1,4) = 29.60$ ,  $P = 0.0055$ ]. Thus, CPG35348 was most potent between 25 and 130 min after its administration, and 4-fold more potent to antagonize baclofen and SKF97541 than GHB and GBL (Fig. 5).

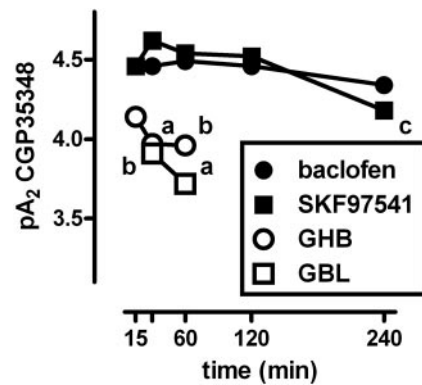
## Discussion

GABA<sub>B</sub> receptors seem to play an important role in the effects of GHB; however, the effects of GHB, although similar, are not identical to those of the prototypical GABA<sub>B</sub> receptor agonist baclofen (e.g., Carter et al., 2009). The main finding of the present study is that the GABA<sub>B</sub> receptor antagonist CPG35348 was significantly more potent to antagonize behavioral effects of the GABA<sub>B</sub> receptor agonists



**Fig. 4.** Time-dependent attenuation by CGP35348 of the response rate-decreasing effects of SKF97541 in pigeons ( $n = 5-6$ ) (left, top to bottom, and right, top and middle). CGP35348 or saline was injected 10 min before SKF97541, and key peck responding was measured at different times after the agonist was injected (range, 15–240 min). For each time interval, the rate of key peck responding, expressed as a percentage of saline control, is plotted as a function of dose after pretreatment with saline (●) or different doses of CGP35348 [○, △, ▽, data comprising the linear portion of the dose-response curves, and fitted with log-linear regression lines; gray-filled symbols, data obtained with the same doses of CGP35348 as the corresponding open symbols, but not part of the linear portion of the dose-response curves, and not used in the regression calculations (see “Data Analysis”). Symbols represent mean  $\pm$  S.E.M.; if not shown, S.E.M. values are contained by the symbol. Bottom right, Schild regression plots for the ability of CGP35348 to antagonize, at different times after its administration (in minutes), the response rate-decreasing effects of SKF97541. Dose ratios are the  $ED_{50}$  values of each agonist in the presence of CGP35348 (3.2–32 mg/kg) divided by the  $ED_{50}$  value of the agonist after pretreatment with saline. Data obtained at each time interval could be fitted with a regression line with a slope of  $-1$ . Calculated from these regression lines, the  $pA_2$  value of CGP35348 to antagonize SKF97541 at the different intervals ranged from 4.18 (95% confidence limits, 3.92–4.43) at 240 min after SKF97541 to 4.63 (4.17–5.09) at 30 min after SKF97541 (i.e., 250 and 40 min after CGP35348, respectively).

baclofen and SKF97541 than those of GHB and its precursor GBL. Schild plots with slope values not significantly different from  $-1$ , consistent with simple competitive antagonism, yielded a  $pA_2$  value of CGP35348 to antagonize baclofen and SKF97541 [i.e., 4.5 (4.4–4.7)] that was similar to *in vitro*  $pA_2$  values reported previously for CGP35348 to antagonize baclofen (i.e., 4.3–4.7) (Kerr et al., 1993; Olanas and Onali, 1999), but that was approximately 4-fold higher than its  $pA_2$  value to antagonize GHB and GBL [i.e., 3.9 (3.7–4.2)]. These findings are consistent with previous observations of differential antagonism by CGP35348 of the discriminative stimulus effects of baclofen and GHB in rats (Carter et al., 2006) and of the cataleptic effects of baclofen and GHB in mice (Koek et al., 2007b), and extend them to effects on operant responding in pigeons. Together, these data are further evidence that GABA<sub>B</sub> receptors mediate many behavioral effects of GHB, but they also suggest that the underlying GABA<sub>B</sub> mechanisms differ from those mediating the effects of prototypical GABA<sub>B</sub> receptor agonists such as baclofen.



**Fig. 5.** Antagonism by CGP35348 of the rate-decreasing effects of different agonists at different times after their administration.  $pA_2$  values are plotted for each agonist at the times it had rate-decreasing effects. CGP35348 was approximately 4-fold less potent to antagonize the effects of GHB and GBL than to antagonize the effects of baclofen and SKF97541. CGP35348 was approximately 2-fold less potent to antagonize SKF97541 at 240 min after its administration than at shorter intervals. a,  $P < 0.05$  compared with baclofen and SKF; b,  $P < 0.05$  and  $< 0.10$  compared with SKF97541 and baclofen, respectively; c,  $P < 0.05$  compared with SKF97541 at all shorter intervals.

Additional evidence that the GABA<sub>B</sub> receptor mechanisms underlying the effects of GHB and baclofen are not identical has recently been obtained in interaction studies with NMDA antagonists. The NMDA antagonist PCP and GHB enhance each other's discriminative stimulus effects, but PCP and baclofen do not, suggesting that the mechanisms underlying these effects of GHB and baclofen are differentially modulated by the glutamatergic system with which PCP interacts (Koek et al., 2007a). The recent finding that PCP and other antagonists at the NMDA subtype of glutamate receptors enhance the cataleptic effects of GHB but not those of baclofen (Koek and France, 2008) provides further evidence that the GABA<sub>B</sub> receptor systems mediating the effects of GHB and baclofen are differentially modulated by glutamate.

Recent electrophysiological studies offer further evidence of differing effects of GHB and baclofen. At concentrations described as clinically relevant, GHB disinhibits and baclofen inhibits ventral tegmental dopamine neurons (Cruz et al., 2004). It has been suggested that GHB is more likely to activate the dopamine system implicated in addiction, whereas baclofen, which may be useful to reduce a relapse to taking cocaine (Weerts et al., 2007), may have more pronounced anticraving effects (Cruz et al., 2004). GHB and baclofen differ also in their effects on neurotransmission at glutamate receptors (Li et al., 2007). GHB and baclofen both inhibited currents elicited by NMDA and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and their effects could be reversed by the GABA<sub>B</sub> receptor antagonist CGP62349. However, GHB was more potent to inhibit NMDA-elicited currents, whereas baclofen was more potent to inhibit  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-elicited currents (Li et al., 2007). These latter findings, together with previous *in vivo* findings (Koek et al., 2007a; Koek and France, 2008), suggest a more prominent role for NMDA receptors in the GABA<sub>B</sub> receptor-mediated effects of GHB. Thus, evidence is emerging that the effects of GHB and baclofen, although in many respects similar, may be mediated by different GABA<sub>B</sub> systems.

There is evidence for functional GABA<sub>B</sub> receptor subtypes (Seabrook et al., 1990; Bonanno and Raiteri, 1992; Lanza et

al., 1993; Fassio et al., 1994; Yamada et al., 1999). Conceivably, differential activity of GHB and baclofen at GABA<sub>B</sub> autoreceptors and heteroreceptors could account for the differential enhancement of their effects by NMDA antagonists, with effects of GHB mediated by glutamatergic GABA<sub>B</sub> heteroreceptors and effects of baclofen by GABA<sub>B</sub> autoreceptors. Differential activity of GHB and baclofen at these receptors could also account for the differential ability of CGP35348 to antagonize their effects. Alternatively, GHB and baclofen may interact differently with the same GABA<sub>B</sub> receptor (e.g., GHB may induce conformational changes in the GABA<sub>B</sub> receptor that differ from those induced by baclofen). Further studies examining the different GABA<sub>B</sub> mechanisms that underlie the effects of GHB and baclofen may help to explain why GHB is effective in treating narcolepsy and is abused, whereas there is no evidence that baclofen is effective in any sleep disorder or that it is abused.

The procedure used in the present study yielded not only quantitative measures of antagonist potency, but also information about the time course of agonist and antagonist effects. GHB and GBL had a more rapid onset and a shorter duration of action than baclofen and SKF97541 to decrease operant response rate in pigeons, in agreement with and extending previous observations in rats (Carter et al., 2004). Because baclofen and SKF97541 had long-lasting effects, pA<sub>2</sub> values for CGP35348 to antagonize these effects could be calculated for injection-test intervals ranging from 15 to 240 min. Changes of pA<sub>2</sub> values over time have been used to provide a description of the duration of action of antagonists (Gerak and France, 2007). From these pA<sub>2</sub> values obtained at different injection-test intervals, it seems that the antagonist potency of CGP35348 was maximal and remained unchanged from 25 to 130 min after its injection. At 240 min, the antagonist potency of CGP35348 decreased, perhaps resulting from it being eliminated. At least 120 min of duration of antagonist action makes CGP35348 suitable for use as a pretreatment in cumulative dosing procedures. In general, cumulative dosing is generally more rapid and economical than single dosing to obtain full dose-response curves. Thus, future studies with CGP35348 in pigeons will use cumulative dosing of agonists.

In summary, the GABA<sub>B</sub> receptor antagonist CGP35348 was significantly more potent to antagonize behavioral effects of the GABA<sub>B</sub> receptor agonists baclofen and SKF97541 than those of GHB and its precursor GBL. This is further evidence that the effects of GHB and prototypical GABA<sub>B</sub> agonists are mediated by GABA<sub>B</sub> receptor mechanisms that are not identical. A better understanding of these mechanisms may help to explain why GHB is effective for treating narcolepsy and is abused, whereas baclofen is not, and this knowledge could lead to more effective medications with fewer adverse effects.

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**Address correspondence to:** Dr. Wouter Koek, Department of Psychiatry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, Mail Code 7792, San Antonio, TX 78229-3900. E-mail: koek@uthscsa.edu

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