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Synthesis of the Nanomolar Photoaffinity $GABA_B$ Receptor Ligand CGP 71872 Reveals Diversity in the Tissue Distribution of $GABA_B$ Receptor Forms

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Abstract—A radioiodinated probe, **[^{125}I]-CGP 71872**, containing an azido group that can be photoactivated, was synthesized and used to characterize $GABA_B$ receptors. Photoaffinity labeling experiments using crude membranes prepared from rat brain revealed two predominant ligand binding species at ~130 and ~100 kDa believed to represent the long ($GABA_B$R1a) and short ($GABA_B$R1b) forms of the receptor. Indeed, these ligand binding proteins were immunoprecipitated using a $GABA_B$ receptor-specific antibody confirming the receptor specificity of the photoaffinity probe. Most convincingly, **[^{125}I]-CGP 71872** binding was competitively inhibited in a dose-dependent manner by cold **CGP 71872**, GABA, saclofen, (−)-baclofen, (+)-baclofen and (L)-glutamic acid with a rank order and stereospecificity characteristic of the $GABA_B$ receptor. Photoaffinity labeling experiments revealed that the recombinant $GABA_B$R2 receptor does not bind **[^{125}I]-CGP 71872**, providing surprising and direct evidence that **CGP 71872** is a $GABA_B$R1 selective antagonist. Photoaffinity labeling experiments using rat tissues showed that both $GABA_B$R1a and $GABA_B$R1b are co-expressed in the brain, spinal cord, stomach and testis, but only the short $GABA_B$R1b receptor form was detected in kidney and liver whereas the long $GABA_B$R1a form was selectively expressed in the adrenal gland, pituitary, spleen and prostate. We report herein the synthesis and biochemical characterization of the nanomolar affinity **[^{125}I]-CGP 71872** and **CGP 71872** $GABA_B$R1 ligands, and differential tissue expression of the long $GABA_B$R1a and short $GABA_B$R1b receptor forms in rat and dog.

Introduction

Amino acids such as glutamic acid, γ-aminobutyric acid (GABA) and glycine are neurotransmitters that bind to specific receptors in the vertebrate nervous system and mediate synaptic transmission. Of these amino acids, GABA is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system. The biological activities of GABA are mediated by three types of GABA receptors: ionotropic $GABA_A$ receptors, metabotropic $GABA_B$ G protein-coupled receptors, and ionotropic $GABA_C$ receptors. Each type of receptor has its own characteristic molecular structure, pattern of gene expression, agonist and antagonist mediated spectrum of physiological activities.

Biology

$GABA_B$ receptors were first distinguished by Hill and Bowery.[1] Based on their anatomical location and physiological functions, $GABA_B$ receptors act presynaptically as autoreceptors and heteroreceptors and also act postsynaptically. Functionally, $GABA_B$ receptors couple through G proteins to neuronal K^+ or Ca^{2+} channels. Receptor activation leading to increased K^+ conductance is responsible for late inhibitory postsynaptic potentials (IPSPs), whereas receptor mediated decrease in Ca^{2+} conductance results in presynaptic inhibition of transmitter release. There is also evidence that $GABA_B$ receptor activation leads to inhibition or potentiates adenylyl cyclase activity, although the functional significance of

Key words: CGP 71872; $GABA_B$ receptor; GABA; photoaffinity labeling.

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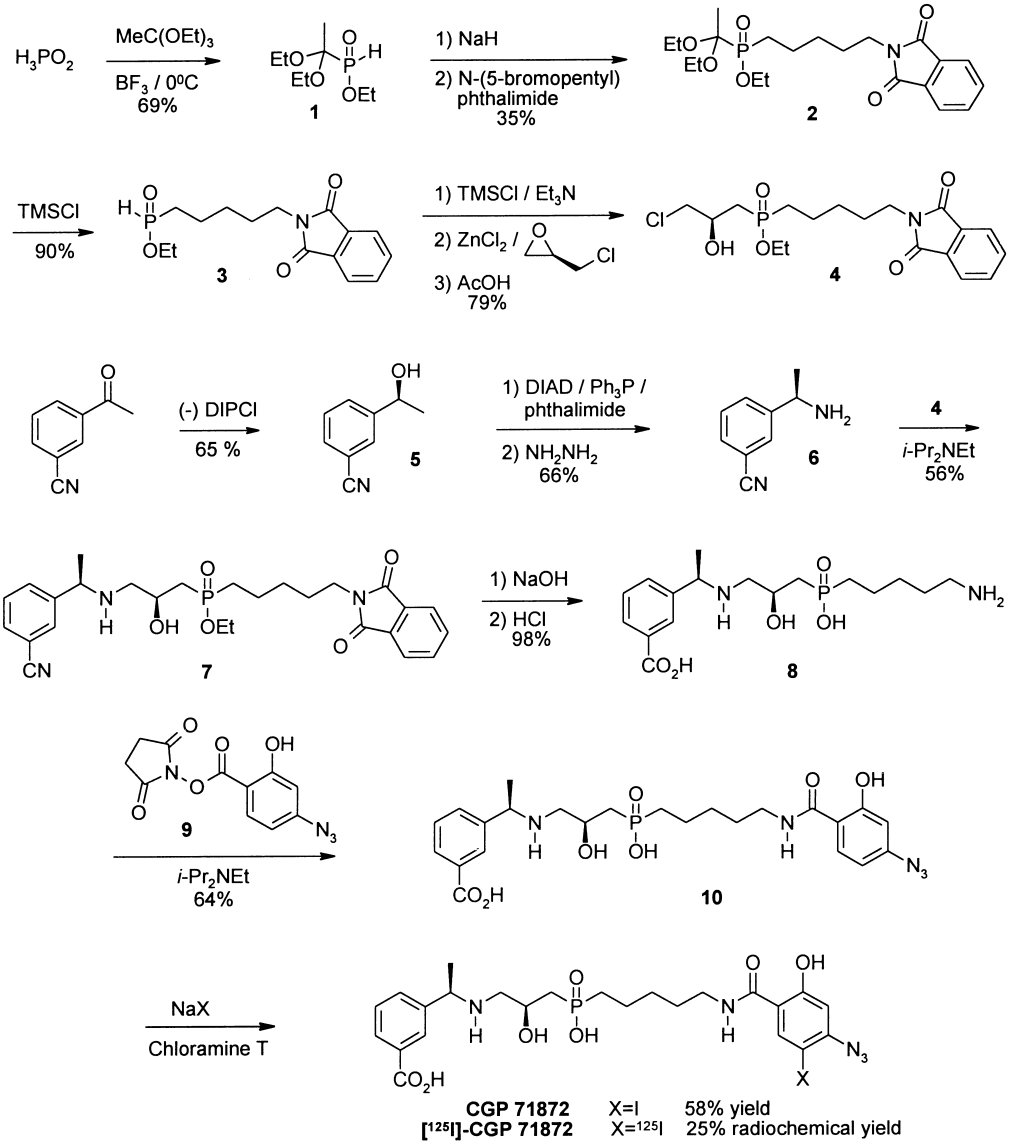
these second messenger changes remains unclear. The molecular cloning of two members of the rat $GABA_B$ receptor family, $GABA_BR1a$ and $GABA_BR1b$, was finally achieved by expression cloning by Kaupmann et al.[2] using high-affinity $GABA_B$ **CGP 64213** and **CGP 71872** receptor antagonists. This pivotal paper, however, did not report the synthesis of these powerful $GABA_B$ receptor probes. In studying the recombinant $GABA_BR1$ receptors and a $GABA_B$-like orphan GPR 51 receptor, now identified as $GABA_BR2$, we and others observed that both receptors are functionally inactive when expressed individually in multiple heterologous expression systems, and that the functional $GABA_B$ receptor is a heterodimer of $GABA_BR1$ and $GABA_BR2$ subtypes.[3–7] However, our understanding of the molecular basis of $GABA_B$ heterodimer activation and ligand binding is still rudimentary. With the aim of clarifying the pharmacological properties and therapeutic potential of the functional $GABA_B$ receptor, we report herein the detailed synthesis of **[^{125}I]-CGP 71872**, the characterization of its pharmacological specificity and the tissue distribution of the $GABA_B$ receptors in rat and dog as determined by photoaffinity labeling studies.

Chemistry

[^{125}I]-CGP 71872 was derived from structure–activity relationship studies starting from the $GABA_B$ receptor agonist 3-aminopropylphosphinic acid, a close analogue of γ-aminobutyric acid.[2,8] It was designed to be a photoaffinity probe: when bound to the receptor, the aryl azide function can be activated by photolysis (360 nm) to generate a highly reactive nitrene intermediate which can then react to create a covalent bond with the surrounding protein.

[^{125}I]-CGP 71872 was prepared in a manner similar to its synthesis summarized in a patent,[9–11] with some modifications (Scheme 1). The preparation of ethyl (1,1-diethoxyethyl)phosphinate **1** from phosphinic acid and



Scheme 1. Synthesis of **CGP 71872**.

triethyl orthoacetate under Lewis acid catalysis using the reported procedures[10,12] gave low and non-reproducible yields of **1**, along with varying amounts of diethyl (1,1-diethoxyethyl)phosphonate $(EtO)_2CMePO(OEt)_2$. Reducing the temperature and the time of the reaction greatly improved the preparation. Deprotonation of **1** with sodium hydride and reaction with *N*-(5-bromopentyl)phthalimide afforded **2**, which was deprotected with chlorotrimethylsilane to give the phosphinic acid **3**. Treatment of **3** with chlorotrimethylsilane and triethylamine yielded a trivalent silylated phosphonite that is very sensitive to oxidation and should be filtered under argon and concentrated with a high vacuum pump. Reaction with fused zinc chloride and epichlorohydrin at 80°C (in the absence of solvent) and hydrolysis of the silyl ether with acetic acid yielded the intermediate **4**. The (cyanophenyl)-ethylamine **6** was prepared via a new route in three steps by a chiral reduction with (−)-B-chlorodiisopinocampheylborane[13,14] followed by a Mitsunobu reaction with phthalimide and deprotection to the amine.[15–17] Substitution of the chlorine of **4** with the primary amine **6** afforded the secondary amine **7**. The nitrile was hydrolyzed with NaOH and the phthalimide removed with concentrated HCl to yield the amine **8**. This is reacted with the commercially available *N*-hydroxysuccinimidyl-4-azidosalicylic acid **9** to give the intermediate **10** which is iodinated to **CGP 71872** with chloramine T and sodium iodide.

Results and Discussion

Since the ligand binding properties of **CGP 71872** have not been reported in detail previously, its characterization is given herein. With the high specific activity of iodine (2200 Ci/mmol) attached to the ligand, the tissue distribution and the therapeutic potential of $GABA_B$ receptors has also been defined.

[^{125}I]-CGP 71872 labels $GABA_BR1a$ and $GABA_BR1b$ receptors, but not $GABA_BR2$ receptors

Photolabeling experiments were carried out to characterize the substrate specificity of **[^{125}I]-CGP 71872**. Upon exposure to UV light, **[^{125}I]-CGP 71872** photolabeled major bands at ~130 and ~100 kDa in crude membranes prepared from rat brain at the predicted molecular mass reported for $GABA_BR1a$ and $GABA_BR1b$ receptors[2] (Fig. 1A). Indeed, $GABA_B$ receptor GB-1713.1 and GB-1713.2 polyclonal antibodies but not preimmune sera were able to immunoprecipitate these ligand binding species (Fig. 1B) confirming the receptor specificity of the ligand. Similar ~130 and ~100 kDa ligand binding species were observed in dog brain membranes (Fig. 1C). Faintly labeled species at approximately twice the molecular mass of the receptor monomers in rat and dog brain membranes suggest that $GABA_BR1$ receptors may undergo homodimerization,[18] also reported for the structurally related metabotropic glutamate receptors.[19]

With the identification of the functional $GABA_B$ receptor which requires the coexpression and heterodimerization of $GABA_BR1$ and $GABA_BR2$ receptors,[3–7] it seemed important to test whether the potent **CGP 71872** antagonist can

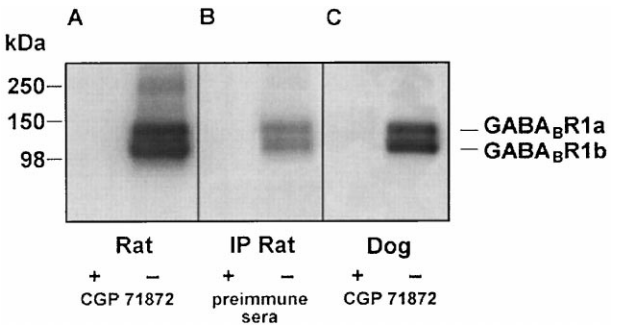


Figure 1. [^{125}I]-CGP 71872 labels $GABA_BR1a$ and $GABA_BR1b$ receptors. (A) Membranes (25 μg) derived from rat brain were photolabeled with **[^{125}I]-CGP 71872** in the absence (−) and presence of 1 μM **CGP 71872** (+) and visualized by autoradiography. (B) $GABA_BR1a$ and $GABA_BR1b$ receptors were immunoprecipitated using $GABA_B$ receptor-specific antibodies 1713.1–1713.2 (−) but not preimmune serum (+). (C) **[^{125}I]-CGP 71872** labeled dog brain $GABA_B$ receptor forms in the absence (−) and presence (+) of 1 μM CGP 71872.

label the $GABA_BR2$ receptor. Photoaffinity labeling experiments using 100 nM final concentration of **[^{125}I]-CGP 71872** revealed that the recombinant $GABA_BR2$ receptor[3–7] (when expressed in COS-7 cells) does not bind the ligand, even though its expression was confirmed by immunoblot analysis (data not shown). This suggests that **[^{125}I]-CGP 71872** is a $GABA_BR1$ receptor selective ligand and that the $GABA_BR1$ subunit is primarily responsible for ligand binding to the $GABA_BR1$–$GABA_BR2$ heterodimer.

The abundant brain expression of $GABA_BR1a$ and $GABA_BR1b$ receptors suggests that both the long and short $GABA_B$ receptors contribute to the reported GABA and baclofen activated G protein-gated inwardly rectifying K^+ channels and/or Ca^{2+} channels which have been implicated in hippocampal long-term potential, absence epilepsy, slow-wave sleep, muscle relaxation and antinociception activities.[8,20]

CGP 71872 exhibits nanomolar affinity for $GABA_BR1a$ and $GABA_BR1b$ receptors

Displacement of **[^{125}I]-CGP 71872** binding to membranes prepared from whole brain in filtration-binding experiments was in the appropriate rank order for GABAergic ligands: **CGP 71872** (K_i 0.48 ± 0.08 nM (n = 4) (mean ± standard deviation)) > GABA (2.50 ± 0.06 μM (n = 3)) > saclofen (156.13 ± 45.88 μM (n = 3)) > (−)-baclofen (0.52 ± 0.04 mM (n = 3)) > (+)-baclofen (1.14 ± 0.31 mM (n = 3)) > L-glutamic acid (4.91 ± 0.30 mM (n = 3)) (Fig. 2(a)). The affinity of baclofen in whole brain tissue is lower compared to previous studies using brain cortex tissues.[2] One explanation might be that the baclofen affinity in whole brain reflects binding to multiple and pharmacologically different $GABA_B$ receptor subtypes that are not expressed in brain cortex. $GABA_BR1$ and $GABA_BR2$ receptors are differentially expressed in brain, especially in the basal ganglia, where $GABA_BR1$ RNA expression is high whereas $GABA_BR2$ RNA expression is low.[3–7] Since the $GABA_BR1$ receptor requires the coexpression of the $GABA_BR2$ receptor for membrane targeting and the formation of the functional $GABA_B$ receptor,[3–7] this

suggests the existence of other $GABA_B$ receptors in the basal ganglia that would enable the formation of a functional $GABA_B$ receptor heterodimer. Further, $GABA_BR1$ receptor isoforms exhibit differential cellular and extrasynaptic localization in brain, suggesting different pharmacology and functions.[21,22] Photoaffinity labeling experiments showed that the long $GABA_BR1a$ and short $GABA_BR1b$ receptor forms differ slightly in their intensity of labeling, suggesting that the short form may have a higher affinity for **CGP 71872** or is more highly expressed compared to the long $GABA_BR1a$ receptor (Fig. 2(b)). Taken together, **CGP 71872** is the highest affinity ligand so far described with a K_i of ~0.5 nM at $GABA_BR1a$ and $GABA_BR1b$ receptors.

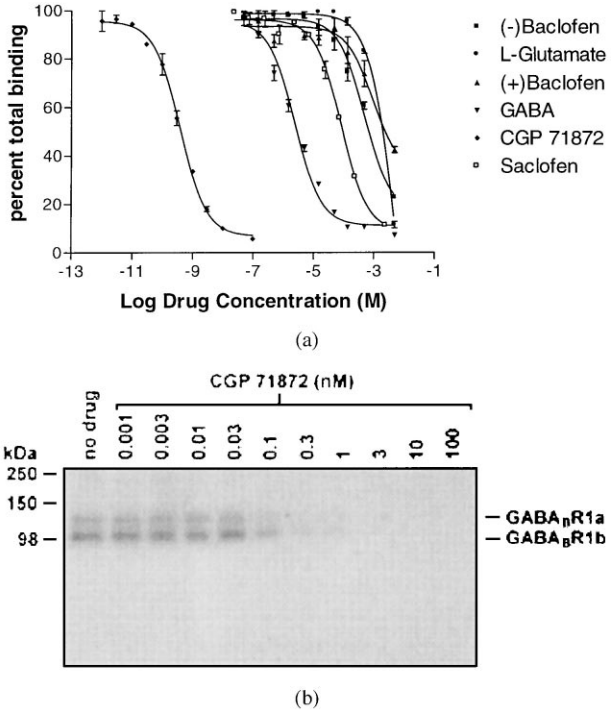


Figure 2. Ligand binding properties of rat brain $GABA_BR1a$ and $GABA_BR1b$ receptors. (a) Pharmacological characterization of $GABA_B$ receptors using filtration-competition binding experiments. (b) **CGP 71872** competition of **[^{125}I]-CGP 71872** binding in photoaffinity assays.

Peripheral tissue expression of $GABA_BR1a$ and $GABA_BR1b$ receptors

There is clear evidence of the functional importance of $GABA_B$ receptors in the brain, but the lack of high-affinity $GABA_B$ receptor-specific ligands and low peripheral expression have made it difficult to establish a clear role for this receptor in GABAergic mechanisms outside the CNS.[23,24] The potential importance of $GABA_B$ receptors in peripheral GABAergic functions is now supported by photoaffinity labeling studies using the nanomolar **CGP 71872** ligand in tissues prepared from rat and dog.

CGP 71872 labeling studies revealed long and short $GABA_B$ receptor proteins in stomach and testis tissues (Table 1, Fig. 3). The known involvement of $GABA_B$ receptors in stomach physiology has been to mediate gut relaxation by inhibition of postganglionic cholinergic nerves.[23] The role(s) of $GABA_B$ receptors outside a nervous based mechanism of action is less clear. In the isolated stomach, GABA has been localized to secretory granules of gastric mucosal epithelium and shown to modulate gastric acid secretion likely via $GABA_A$ receptors.[24,25] The morphological feature of $GABA_B$ receptor-positive cells in the gastrointestinal mucosa[26]

Table 1. Summary of differential expression of long and short $GABA_B$ receptor forms in rat and dog tissues[a]

	Rat		Dog		
	130 kDa long isoform	100 kDa short isoform	130 kDa long isoform	100 kDa short isoform	Rat/dog film exposure time
Spinal cord	+ +	+ +	+ +	+ +	h–1 day
Brain	+ + +	+ + +	+ + +	+ + +	h–1 day
Heart	ND	ND	—	—	4 weeks
Lung	ND	ND	ND	ND	4 weeks
Muscle	ND	ND	ND	ND	4 weeks
Liver	ND	+	ND	ND	2 weeks
Spleen	+	ND	+	ND	4 weeks/2 weeks
Small intestine	ND	ND	ND	ND	4 weeks
PBL	ND	ND	—	—	4 weeks
Pituitary	+	ND	+	ND	4 weeks/2 weeks
Adrenal gland	+	ND	+	ND	4 weeks
Prostate	+	ND	+	ND	4 weeks/2 weeks
Testis	+	+	—	—	1 week
Ovary	ND	ND	—	—	4 weeks
Trachea	ND	ND	—	—	4 weeks
Pancreas	ND	ND	ND	ND	4 weeks
Colon	ND	ND	—	—	4 weeks
Kidney	ND	+ +	+	+	1/3 weeks
Stomach	+	+	ND	ND	1 week
Thyroid	ND	ND	—	—	4 weeks
Thymus	ND	ND	—	—	4 weeks
Bone marrow	ND	ND	—	—	4 weeks
Lymph node	ND	ND	—	—	4 weeks

[a] ND: not detected; + + +: high expression; + +: intermediate expression; +: low expression; —: tissue not tested.

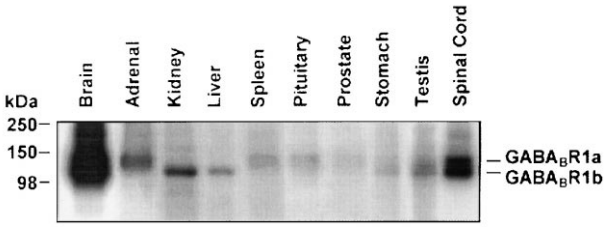


Figure 3. Differential expression of $GABA_BR1a$ and $GABA_BR1b$ receptors in rat tissues. Photoaffinity **[^{125}I]-CGP 71872** labeling of $GABA_B$ receptor forms in multiple tissues. The long $GABA_BR1a$ and short $GABA_BR1b$ receptors are indicated. Autoradiogram exposure times are as follows: brain, 1 week; kidney, 3 weeks; stomach, 3 weeks; liver, 10 weeks; spleen, 10 weeks; pituitary, 10 weeks; prostate, 10 weeks; testis, 9 weeks; spinal cord, 1 week; adrenal gland, 12 weeks.

would, however, support the expression of $GABA_B$ receptors in the stomach such that both $GABA_A$ and $GABA_B$ receptors may be involved in mediating the local hormone actions of GABA on the secretion of gastrin and somatostatin.[27,28]

A role for $GABA_B$ receptors in the testis has not been described and is unknown. GABA has been detected in spermatozoa and presumably acts via $GABA_A$ receptors to regulate the motility of the spermatozoon.[24] Recent biochemical findings have shown GABA modulation of androgen secretion in testicular tissue, probably by a direct action on endocrine cells of the testis,[29] but the receptors involved remain to be determined.

The long form of the receptor, $GABA_BR1a$, was expressed absent of the short $GABA_BR1b$ receptor in restricted peripheral tissues (Table 1, Fig. 3). **[^{125}I]-CGP 71872** labeled $GABA_BR1a$ in endocrine tissues such as pituitary, adrenal gland, prostate, and epithelial tissues such as spleen. GABA and glutamic acid decarboxylase have been shown to be localized to both anterior and intermediate lobes of the pituitary,[24] suggesting that $GABA_BR1a$ receptor activation may contribute to GABA mediated decrease in corticotrophin-releasing hormone, prolactin-releasing factor, luteinizing hormone levels including increasing androgen production.[30] Indeed, baclofen, a selective $GABA_B$ receptor agonist, exhibits robust inhibition of pituitary secretions.[30] We observed $GABA_BR1a$ expression in the adrenal gland. Although its role in local hormone actions is not known, $GABA_B$ receptors have been reported to modulate acetylcholine-evoked catecholamine release.[24] A role for $GABA_BR1a$ receptors in prostate and spleen physiology is less clear. Much of the evidence suggests a role for the $GABA_A$ receptor in the immune modulatory role of GABA on the proliferation of thymic cells and of spleen lymphocytes.[24] The localization of $GABA_BR1a$ in spleen suggests that this $GABA_B$ receptor form may be important as well. No information is available about $GABA_B$ receptors in the prostate although its expression was detected by **[^{125}I]-CGP 71872** binding.

[^{125}I]-CGP 71872 revealed selective expression of the short $GABA_BR1b$ receptor form in exocrine and epithelial tissues such as kidney and liver (Table 1, Fig. 3). GABA is believed to have a possible role in renal ammoniagenesis and in the function of proximal tubular cells.[23] The biological actions of GABA in these regions are likely mediated via $GABA_A$ receptors,[31] although baclofen binding studies have indicated expression of $GABA_B$ receptors in the kidney as well.[32] The demonstration of high-affinity GABA uptake sites on hepatocytes, localization of GABA and GAD on these cells, and demonstration of bicuculline-sensitive ($GABA_A$ receptor) GABA binding support a GABAergic component to bile secretion.[24,33] The localization of $GABA_BR1b$ to this exocrine tissue may suggest an influence on bile secretion as well.

We showed for the first time differential peripheral tissue expression of the long $GABA_BR1a$ and short $GABA_BR1b$ receptor forms. Because the functional $GABA_B$ receptor is a $GABA_BR1$–$GABA_BR2$ heterodimer, this suggests that possibly $GABA_BR1a$–$GABA_BR2$ and $GABA_BR1b$–$GABA_BR2$ heterodimers mediate different physiological functions, thus accounting for the diverse biological activities of GABA. Clearly more detailed in situ hybridization studies are needed to confirm that the expression of $GABA_B$ receptors is not confined to nerve tissue within the periphery. $GABA_B$ receptors were not detected in a number of tissues: trachea, heart, lymph node, peripheral blood leukocytes, bone marrow, skeletal muscle, small intestine, colon, ovary, pancreas, thyroid, thymus, and lung. One explanation for the effects of the $GABA_B$ receptor agonists such as baclofen on the physiology of some of these organs may be that it involves central $GABA_B$ receptors or that other $GABA_B$ receptor forms not labeled by **CGP 71872** remain to be discovered at the molecular level.

Experimental

Synthesis of [^{125}I]-CGP 71872; general comments

N-Hydroxysuccinimidyl-4-azidosalicylic acid was purchased from Pierce. Many intermediates in the synthesis are hard to reveal on TLC (silica gel): they have to be spotted heavily (a whole drop of reaction mixture from a pipette), checked by UV and revealed with iodine followed by a cerium/molybdate dip to give, sometimes, only faint and diffuse spots. The chemical shifts of the protons for compounds **6**, **7**, **9** and **CGP 71872** also vary from one sample to another due to pH variation and/or contamination by solvents or impurities such as *i*-$Pr_2NEt{\cdot}AcOH$ or NH_4OAc. Mass spectrometry was used often to follow reactions and to identify the product distribution.

Ethyl (1,1-diethoxyethyl)phosphinate 1. Hypophosphorous acid was concentrated down, at 40°C under high vacuum overnight, to a yellow syrup which crystallized in the fridge.[34] At 5°C, $BF_3{\cdot}Et_2O$ (10 mL) was added to triethyl orthoacetate (450 mL) and the mixture was cooled down to −20°C. A solution of concentrated hypophorous acid (39 g, 417 mmol) in THF (200 mL) was then added slowly and the mixture was warmed to 0°C for 2 h (until all the solid H_3PO_2 was dissolved; the reaction could be monitored by TLC using acetone:toluene 2:3 as eluent). 0.4 M NaH_2PO_4 was then added slowly and the product was extracted into *i*-PrOAc, washed with brine, dried over Na_2SO_4 and concentrated. Distillation at 61°C/0.1 mm Hg yielded 74.35 g of **1** with a purity of 87% (69%

yield). The major impurity co-distilling with **1** was diethyl (1,1-diethoxyethyl)phosphonate. ^{1}H NMR (acetone-d_6) δ 1.17 (6H, t), 1.32 (3H, t), 1.38 (3H, d), 3.68 (4H, m), 4.17 (2H, m), 6.85 (1H, d, J = 540 Hz, PH).

***N*-(5-((1,1-Diethoxyethyl)ethoxyphosphoryl)pentyl)phthalimide 2**. At 0°C, 80% NaH in oil (502 mg, 16.7 mmol) was added to **1** (4.11 g, 80% purity, 15.6 mmol) in THF (80 mL) and the mixture was stirred at room temperature for 4 h. At 0°C, *N*-(5-bromopentyl)phthalimide (4.12 g, 13.9 mmol) was added and the mixture was stirred again at room temperature for 4 h and was quenched by the addition of half saturated NH_4Cl. The product was extracted into *i*-PrOAc, dried over Na_2SO_4 and purified by flash chromatography on silica with acetone:toluene 1:4 to give 2.08 g (35% yield) of **2**. ^{1}H NMR (acetone-d_6) δ 1.14 (6H, 2t), 1.25 (2H, t), 1.40 (2H, d), 1.46 (2H, m), 1.58–1.77 (6H, m), 3.60 (1H, m), 3.65 (4H, 2q), 3.74 (1H, m), 4.08 (2H, m), 7.83 (4H, s). MS (APCI, pos.) 380.2 (M−OEt).

***N*-(5-(Ethoxyphosphoryl)pentyl)phthalimide 3**. Trimethylchlorosilane (6.6 mL) was added slowly to a solution of **2** (6.79 g, 16.0 mmol) in CH_2Cl_2:MeOH 9:1 (100 mL) at 0°C and the mixture was stirred at room temperature overnight. After addition of half saturated NH_4Cl, the product was extracted into *i*-PrOAc, dried over Na_2SO_4 and purified by flash chromatography on silica with acetone:toluene:AcOH 50:50:1 to give 4.40 g (90% yield) of **3** as a yellow oil. ^{1}H NMR (acetone-d_6) δ 1.28 (3H, t), 1.47 (2H, m), 1.59 (2H, m), 1.71 (4H, m), 3.65 (2H, t), 3.97–4.09 (2H, m), 6.98 (1H, d, J = 526 Hz, PH), 7.85 (4H, s). MS (APCI, pos.) 310.9, 309.9 (M + 1), 281.9, 264.0 (M−OEt).

***N*-(5-((3-Chloro-2-(*R*)-hydroxypropyl)ethoxyphosphoryl)-pentyl)phthalimide 4**. A solution of **3** (4.92 g, 15.9 mmol) in ether (50 mL) was cooled to 0°C under argon. Et_3N (5.0 mL, 2.3 equiv) was added, followed by TMSCl (4.0 mL, 2 equiv) dropwise and the mixture was stirred at room temperature for 3.5 h. THF (30 mL) was added and the triethylamine hydrochloride was removed by filtration under argon. The oxygen sensitive phosphine intermediate was concentrated to dryness under high vacuum and then fused $ZnCl_2$ (990 mg, 0.5 equiv) and (*R*)-(−)-epichlorohydrin (2.0 g, 1.4 equiv) were added quickly while maintaining a positive pressure of argon. After heating to 80°C for 3 h under argon, the reaction mixture was dissolved in MeOH:AcOH 35:1 (70 mL) and was stirred at room temperature for 3 days to cleave the silyl ether. Evaporation and flash chromatography of the residue on silica with MeOH:CH_2Cl_2 1:19 afforded 5.03 g of **4** (79% yield). ^{1}H NMR (acetone-d_6) δ 1.27 (3H, t), 1.45 (2H, m), 1.62 (2H, m), 1.70 (2H, m), 1.80 (2H, m), 1.90–2.13 (2H, m), 3.57–3.70 (4H, m), 4.02 (2H, 2q), 4.13–4.25 (1H, m), 4.77 (1H, s, OH), 7.84 (4H, s). MS (APCI, pos.) 401.9 (M + 1).

(*S*)-1-(3-Cyanophenyl)ethanol 5.[13,14] To a solution of (−)-B-chlorodiisopinocampheylborane (20 g, 2 equiv) in THF (100 mL) at −25°C was added slowly a solution of 3-acetylbenzonitrile (5.00 g, 34.4 mmol) in THF (60 mL) and the mixture was warmed slowly to −4°C over 1 h. Acetaldehyde (7.7 mL) was then added and the mixture was stirred at room temperature overnight. 10% Diethanolamine was added and the product was extracted into *i*-PrOAc, washed with 1 N HCl and brine, dried over Na_2SO_4 and concentrated to dryness. Flash chromatography on silica with EtOAc:toluene 1:5 afforded 3.25 g (65% yield) of **10** with an enantiomeric excess of 97% (determined from the ^{1}H NMR of its Mosher esters). ^{1}H NMR (acetone-d_6) δ 1.42 (3H, d), 4.46 (1H, d, OH), 4.93 (1H, m), 7.52 (1H, dd), 7.62 (1H, d), 7.72 (1H, d), 7.78 (1H, s).

***N*-(1-(*R*)-(3-Cyanophenyl)ethyl)phthalimide.** At 0°C, diisopropyl azodicarboxylate (4.2 mL) was added dropwise to a solution of **5** (2.17 g, 14.7 mmol), triphenylphosphine (5.60 g) and phthalimide (3.13 g) in THF (100 mL) and the mixture was warmed to room temperature slowly and stirred at that temperature overnight.[15–17] Half-saturated NH_4Cl was added and the product was extracted into *i*-PrOAc, washed with 2 N HCl and brine and dried over Na_2SO_4. Purification by flash chromatography on silica with EtOAc:toluene 1:19 and trituration of the solid in ether:hexane 1:1 (50 mL) afforded 2.83 g (70% yield) of a white solid. ^{1}H NMR (acetone-d_6) δ 1.93 (3H, d), 5.60 (1H, q), 7.58 (1H, dd), 7.68 (1H, d), 7.80 (1H, d), 7.83 (4H, s), 7.90 (1H, s).

(*R*)-1-(3-Cyanophenyl)ethylamine 6. *N*-(1-(*R*)-(3-Cyanophenyl)ethyl)phthalimide (2.83 g, 10.24 mmol) and hydrazine hydrate (1.03 g, 2 equiv) were heated to reflux in ethanol (50 mL) for 1.6 h. 2 M Na_2CO_3 and *i*-PrOAc were added and the solid dissolved by heating. The products were extracted into *i*-PrOAc, dried over Na_2SO_4 and concentrated. The solid was triturated with ether:hexane 1:1 and filtered. The filtrate was concentrated and distilled on a Kugelrohr apparatus under 15 mm Hg to give 1.41 g (94% yield) of **6**. $[\alpha]_D^{25}$ 28.0 (*c* 1.3, EtOH). ^{1}H NMR ($CDCl_3$) δ 1.37 (3H, d), 4.17 (1H, q), 7.40 (1H, dd), 7.50 (1H, d), 7.59 (1H, d), 7.65 (1H, s).

***N*-(5-((3-(1-(*R*)-(3-Cyanophenyl)ethylamino)-2-(*S*)-hydroxypropyl)hydroxyphosphoryl)pentyl)phthalimide 7**. A mixture of **4** (160 mg, 0.4 mmol), **6** (64 μL) and diisopropylethylamine (83 μL) in EtOH (500 μL) was heated to 80°C for 1 day and 100°C for 2 days in a glass bomb. Saturated $NaHCO_3$ was added and the product was extracted into *i*-PrOAc, washed with brine, dried over Na_2SO_4 and purified by flash chromatography on silica with MeOH:CH_2Cl_2 3:97 and NH_4OH:MeOH:CH_2Cl_2 1:10:75 to give 114 mg (56% yield) of **7**. ^{1}H NMR (methanol-d_4) δ 1.27 (3H, 2t), 1.37 (3H, d), 1.41 (2H, m), 1.60 (2H, m), 1.68 (2H, m), 1.82 (2H, m), 1.86–2.05 (2H, m), 2.42 (1H, m), 2.52 (1H, m), 3.67 (2H, t), 3.83 (1H, q), 3.88–4.05 (3H, m), 7.49 (1H, dd), 7.58 (1H, d), 7.66 (1H, d), 7.73 (1H, s), 7.78 (2H, m), 7.84 (2H, m). MS (APCI, pos.) 512.2 (M + 1).

***N*-(5-((3-(1-(*R*)-(3-Carboxyphenyl)ethylamino)-2-(*S*)-hydroxypropyl)hydroxyphosphoryl)pentyl)phthalimide.** A mixture of **7** (2.24 g, 4.37 mmol), 10 N NaOH (2.6 mL), ethanol (5 mL) and water (8 mL) was heated to 80°C overnight (the reaction could be followed by TLC using MeOH:CH_2Cl_2 1:1 as eluent). 2 N HCl (13 mL) was then added at 0°C and the solvents were coevaporated with toluene. The product was dissolved in MeOH, filtered, concentrated, stripped again with toluene and used as such in the next step. ^{1}H NMR (methanol-d_4) δ 1.50 (2H, m), 1.58–1.76 (9H, m), 1.85–2.03 (2H, m), 3.00 (2H, m), 3.35

(2H, t), 4.23 (1H, br s), 4.50 (1H, m), 7.42 (1H, d), 7.52 (1H, dd), 7.58 (2H, m), 7.75 (1H, d), 7.94 (1H, d), 8.07 (1H, d), 8.18 (1H, s). MS (APCI, neg.) 501.2 (M−1), 371.0, 164.3.

3-(1-(*R*)-((3-((5-Aminopentyl)hydroxyphosphoryl)-2-(*S*)-hydroxypropyl)amino)ethyl)benzoic acid, dihydrochloride 8. *N*-(5-((3-(1-(*R*)-(3-Carboxyphenyl)ethylamino)-2-(*S*)-hydroxypropyl)hydroxyphosphoryl)pentyl)phthalimide (4.37 mmol) was heated in concentrated HCl (15 mL) at 100°C for 2 days (the reaction was monitored by TLC using H_2O:MeOH 1:3 as eluent). The mixture was diluted with H_2O (15 mL) and washed with THF:*i*-PrOAc 1:1 twice to remove the phthalimide. The water was evaporated off and the product dissolved in MeOH, filtered and evaporated to give 1.908 g of **8** as a hygroscopic foam (98% yield for two steps). ^{1}H NMR (methanol-d_4) δ 1.50 (2H, m), 1.58–1.70 (4H, m), 1.73 (3H, d), 1.80 (2H, m), 1.93–2.08 (2H, m), 2.93 (2H, t), 3.05 (2H, d), 4.24 (1H, m), 4.53 (1H, q), 7.60 (1H, dd), 7.77 (1H, d), 8.09 (1H, d), 8.20 (1H, s). MS (APCI, pos.) 373.4 (M + 1).

3-(1-(*R*)-(3-((5-(4-Azido-2-hydroxybenzoylamino)pentyl)-hydroxyphosphoryl)-2-(*S*)-hydroxypropylamino)ethyl)benzoic acid 10. A mixture containing **8** (29 mg, 65 μmol), *N*-hydroxysuccinimidyl-4-azidosalicylic acid **9** (22 mg, 1.2 equiv) and diisopropylethylamine (45 μL) in MeOH (2 mL) was stirred at room temperature overnight in the dark. It was then diluted with CH_2Cl_2 (2 mL), poured on top of a flash chromatography column and eluted with MeOH:CH_2Cl_2:AcOH 50:50:0, then 50:50:1 and 100:0:1 to give 45 mg of a solid containing 50% **10** (yield 64%), along with some *N*-hydroxysuccinimide, *i*-Pr_2NEt·AcOH and solvents. It was used as such in the next step. ^{1}H NMR (methanol-d_4:D_2O) (1.42 (2H, m), 1.52 (4H, m), 1.62 (2H, m), 1.70 (3H, d), 1.70 (1H, m), 1.82 (1H, m), 2.94–3.04 (2H, m), 3.38 (2H, t), 4.18 (1H, m), 4.46 (1H, q), 6.59 (1H, s), 6.68 (1H, d), 7.49 (1H, dd), 7.58 (1H, d), 7.78 (1H, d), 7.95 (1H, d), 7.98 (1H, s). MS (APCI, neg.) 532.7 (M−1), 504.7 (M−N_2).

3-(1-(*R*)-(3-((5-(4-Azido-2-hydroxy-5-iodobenzoylamino)pentyl)hydroxyphosphoryl)-2-(*S*)-hydroxypropylamino)ethyl)-benzoic acid, acetic acid salt CGP 71872. A solution of **10** (112 μmol) in MeOH:H_2O 2:1 (3 mL) was added to NaI (35 mg, 2 equiv) and chloramine T (64 mg, 2 equiv) at 0°C and the mixture was stirred at that temperature for 3 h. Sodium sulfite (2 equiv) in water was then added and the mixture was injected directly onto an HPLC system for purification (Nova-Pak C_{18} cartridge 25×10 cm, flow rate 20 mL/min, detection 300 nm, solvent 53% MeOH, 47% NH_4OAc, 2 g/L). Four peaks could be detected by HPLC: the first peak corresponds to unreacted **10**, the third to the diiodination product and the fourth to **CGP 71872**. The fractions containing **CGP 71872** were concentrated to a small volume and the product was freeze-dried to give 56 mg of a light brown oil containing 84% **CGP 71872** as an acetic acid salt (58% yield) and 16% NH_4OAc. ^{1}H NMR (methanol-d_4:D_2O) δ 1.42 (2H, m), 1.53 (4H, br s), 1.62 (2H, m), 1.70 (3H, d), 1.72 (1H, m), 1.84 (1H, m), 2.02 (3H, s, AcO), 2.93 (1H, dd), 3.01 (1H, dd), 3.36 (2H, t), 4.19 (1H, m), 4.47 (1H, q), 6.80 (1H, s), 7.55 (1H, dd), 7.68 (1H, d), 8.00 (1H, d), 8.05 (1H, s), 8.19 (1H, s). MS (APCI, neg.) 658.6 (M−1), 630.5 (M–N_2−1), 502.5.

[^{125}I]-3-(1-(*R*)-(3-((5-(4-Azido-2-hydroxy-5-iodobenzoylamino)pentyl)hydroxyphosphoryl)-2-(*S*)-hydroxypropylamino)ethyl)benzoic acid [^{125}I]-CGP 71872. To a mixture of **10** (4.5 nmol) in 0.1 M NH_4OAc (37.5 μL) and Na^{125}I (5 mCi, 2200 Ci/mmol; 2.3 nmol) in water (100 μL) was added a solution of chloramine T (9 nmol) in H_2O (75 μL) and the mixture was stirred at room temperature for 10 min before being quenched by 0.1 M Na_2SO_3 (250 μL), diluted with MeOH:H_2O 1:1 (250 μL) and purified quickly by HPLC (Nova-Pak C_{18} column 3.9×150 mm; flow rate 1 mL/min, detection 235 nm, solvent 47% MeOH, 53% NH_4OAc, 2 g/L, containing 0.002% mercaptoethanol). The fractions containing **[^{125}I]-CGP 71872** (1.24 mCi; 25% radiochemical yield) were concentrated to a final volume of 2 mL.

Preparation of membrane fractions

P2 membrane fractions were prepared at 4°C. Tissues were washed twice with cold PBS, collected by centrifugation at 100×*g* for 7 min, and resuspended in 10 mL of buffer A: 5 mM Tris–HCl, 2 mM EDTA containing (1X) protease inhibitor cocktail Complete™ tablets (Boehringer Mannheim), pH 7.4 at 4°C. Tissues were disrupted by polytron homogenization, centrifuged at 100×*g* for 7 min to pellet unbroken cells and nuclei, and the supernatant collected. The resulting pellet was homogenized a second time in 10 mL of buffer A, centrifuged as described above and supernatant fractions saved. The pooled S1 supernatant was centrifuged at high speed (40,000×*g* for 20 min) and the pellet was washed once with buffer A, centrifuged (40,000×*g* for 20 min) and resuspended in buffer A to make the P2 membrane fraction, and stored at −80°C. Protein content was determined using the Bio-Rad Protein Assay Kit according to manufacturer instructions.

Receptor filter-binding assays

Competition of **[^{125}I]-CGP 71872** binding experiments were performed with ~7 μg P2 membrane protein and increasing concentrations of cold ligand (10^{-12}–10^{-3} M). The concentration of radioligand used in the competition assays was 1 nM (final). Each concentration was examined in duplicate and incubated for 2 h at 22°C in the dark in a total volume of 250 μL binding buffer: 50 mM Tris–HCl, 2.5 mM $CaCl_2$ (pH 7.4) with (1X) protease inhibitor cocktail Complete™ tablets. Bound ligand was isolated by rapid filtration through a Brandel 96-well cell harvester using Whatman GF/B filters. Data were analyzed by non-linear least-squares regression using the computer-fitting program GraphPad Prism version 2.01 (San Diego).

Photoaffinity labeling

P2 membranes were resuspended in binding buffer, and incubated in the dark with 1 nM final concentration **[^{125}I]-CGP 71872** (2200 Ci/mmol) in a final volume of 1 mL for 2 h at 22°C. The membranes were centrifuged at 40,000×*g* for 10 min and the pellet was washed in ice-cold binding buffer, centrifuged at 40,000×*g* for 20 min and resuspended in 1 mL of ice-cold binding buffer and

exposed on ice 2 inches from 360 nm ultraviolet light for 10 min. Photolabeled membranes were washed, pelleted by centrifugation and solubilized in sample buffer (50 mM Tris–HCl pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol). Samples were electrophoresed on precast NOVEX 10% Tris–glycine gels, fixed, dried and exposed to Kodak XAR film with an intensifying screen at −70°C.

$GABA_B$ receptor antibodies

Amino acids 212–227 (DVNSRRDILPDYELKLC) and 333–346 (CATLHNPTRVKLFEK) within the N-terminus of the rat $GABA_B$R1a were chosen as immunogenic peptides. Identical sequences are shared with the $GABA_B$R1b receptor, but no significant homology to other G protein-coupled receptors was found after querying the Genbank database. Affinity purified rabbit $GABA_B$ receptor polyclonal antibody 1713.1 (acetyl-DVNSRRDILPDYELKLC-amide) and 1713.2 (acetyl-CATLHNPTRVKLFEK-amide) were custom prepared by Quality Control Biochemicals (Hopkinton, MA). Briefly, immunogenic peptides were incubated with thiol coupling gel (1 mg of peptide for 1 mL of gel) and non-specific sites on the gel blocked. The peptide-coupled gel was incubated with serum, and bound antibody eluted with glycine buffer and dialyzed.

Solubilization and immunoprecipitation of receptors

Membranes were prepared by sonication in buffer A as described. The pellet was resuspended and stirred at 4°C overnight in 2 mL of freshly prepared solubilization buffer consisting of 100 mM NaCl, 10 mM Tris–HCl, pH 7.4, 2% digitonin, and 5 mM EDTA with (1X) protease inhibitor cocktail Complete™ tablets. The homogenate was centrifuged at 40,000×g for 20 min and the solubilized fraction were washed with 10 mL cold buffer C: 100 mM NaCl, 10 mM Tris–HCl, pH 7.4, with protease inhibitors and concentrated in Centriprep 30 cartridges (Amicon). Solubilized receptors were immunoprecipitated during agitation with rabbit preimmune serum or $GABA_B$ receptor antibodies 1713.1 and 1713.2 (1:1000 dilution each) and agarose fixed goat anti-primary IgG (1:40 dilution) (Sigma, MO). The immunoprecipitate was washed six times with 5 volumes of cold buffer C for 20 min, solubilized in SDS sample buffer, sonicated and electrophoresed on 10% Tris–glycine gels as described.

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