ELSEVIER

European Journal of Pharmacology 417 (2001) 177–180

ejp

www.elsevier.nl/locate/ejphar

Pharmacological characterization of homobaclofen on wild type and mutant $GABA_B$1b receptors coexpressed with the $GABA_B$2 receptor

Anders A. Jensen, Bo E. Madsen, Povl Krogsgaard-Larsen, Hans Bräuner-Osborne *

NeuroScience PharmaBiotec Research Centre, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

Received in revised form 14 March 2001; accepted 16 March 2001

Abstract

Homobaclofen (5-amino-3-(4-chlorophenyl) pentanoic acid) is a homologue of the classical $GABA_B$ receptor agonist baclofen. In a recent study, the two enantiomers of this compound were tested in a $GABA_B$ receptor selective [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) binding assay using rat brain homogenate and in an assay of electrically induced contractions of guinea pig ileum. The results from the two tissues did, however, not correlate very well, and in order to further investigate these discrepancies, we have pharmacologically characterized these enantiomers on recombinant wild type and mutant rat $GABA_B$1b receptors coexpressed with rat $GABA_B$2 receptors. The results from this study correlate nicely with the binding data from rat brain. (*R*)-Homobaclofen was shown to act like (*R*)-baclofen albeit with 20-fold less potency, and (*S*)-homobaclofen was inactive on the receptor. The discrepancies between the data obtained in this study and those from the guinea pig ileum model could be ascribed to differences in amino acid sequence or receptor splicing of $GABA_B$ receptors between the two species. Another explanation for the observation is the possible existence of a novel yet uncloned $GABA_B$ receptor in guinea pig iluem. © 2001 Published by Elsevier Science B.V.

Keywords: $GABA_B$ receptor; $GABA_B$1b receptor; Mutagenesis; Baclofen; Homobaclofen; GABA (γ-aminobutyric acid)

1. Introduction

The two $GABA_B$ receptors, $GABA_1$ and $GABA_2$, belong to family C of the G-protein coupled receptor superfamily, which also comprises eight metabotropic glutamate receptors, a calcium-sensing receptor, a subfamily of putative pheromone receptors and three recently cloned orphan receptors (Möhler and Fritschy, 1999; Bräuner-Osborne et al., 2000; Brown, 1999; Tirindelli et al., 1999; Cheng and Lotan, 1998; Bräuner-Osborne and Krogsgaard-Larsen, 2000; Robbins et al., 2000).

In a previous study, homobaclofen (5-amino-3-(4-chlorophenyl)pentanoic acid), a homologue of the classical $GABA_B$ receptor agonist baclofen, was shown to exhibit a quite remarkable functional pharmacological profile in guinea pig ileum as compared to that of baclofen (Karla et al., 1999).

Recently, we have developed a functional assay based on co-transfection of the two $GABA_B$ receptor subunits with the chimeric G-protein Gαq-z5 (Bräuner-Osborne and Krogsgaard-Larsen, 1999). In order to shed light on the remarkable pharmacological properties of (*R*)- and (*S*)-homobaclofen, we have characterized the pharmacological profiles of the compounds on recombinant wild type and mutant $GABA_B$1b receptors coexpressed with the wild type $GABA_B$2 receptor.

2. Materials and methods

2.1. Materials

Culture media, serum, antibiotics and buffers for cell culture were obtained from Life Technologies (Paisley, Scotland). (*R*)-Baclofen, HCl was purchased from RBI (Natick, MA), and (*R*)- and (*S*)-homobaclofen were synthesized in our own laboratory as previously described (Karla et al., 1999). All other chemicals were obtained from Sigma (St. Louis, MO).

* Corresponding author. Tel.: +45-3530-6518; fax: +45-3530-6040.
E-mail address: hbo@dfh.dk (H. Bräuner-Osborne).

0014-2999/01/$ - see front matter © 2001 Published by Elsevier Science B.V.
PII: S0014-2999(01)00918-9

2.2. Construction of mutant $GABA_B$1b receptors

Mutations were made using the Quickchange mutagenesis kit according to the manufacturers instructions (Stratagene, La Jolla, CA). The mutant receptors were sequenced on an ABI 310 using Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington, UK).

2.3. Cell culture and inositol phosphate assays

tsA cells (a transformed human embryonic kidney (HEK) 293 cell line (Chahine et al., 1994)) were maintained at 37°C in humidified 5% CO_2 incubator in Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% calf serum. Briefly, one million tsA cells were plated in a 10-cm tissue culture plate and transfected the following day with 0.7 μg wild type, S130A or S153A $GABA_B$R1b-pCDNA3.1, 3.5 μg $GABA_B$R2-pCDNA3.1 and 0.7 μg Gαq-z5-pCDNAI using Superfect as DNA carrier. The day after transfection, the cells were transferred to a 24-well cluster plate in growth medium containing 1 μCi/ml *myo*-[2-^{3}H]inositol (Amersham, Buckinghamshire, UK). After 16–20 h, the cells were washed in phosphate buffered saline solution (PBS) and incubated for 20 min in PBS supplemented with 0.9 mM $CaCl_2$ and 1.05 mM $MgCl_2$. The cells were then incubated for another 20 min in PBS supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$ and 10 mM LiCl. Finally, the cells were incubated for 40 min in PBS supplemented with 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$, 10 mM LiCl and various concentrations of agonist. The reactions were stopped by exchanging the buffer with 500 μl ice-cold 20 mM formic acid and separation of total [^{3}H]inositol phosphates was carried out by ion-exchange chromatography as previously described (Nanevicz et al., 1996; Bräuner-Osborne et al., 1999).

2.4. Data analysis

Data from the experiments were fitted to the simple mass equation: $R = R_{max}/(1 + (EC_{50}/[A])^n) + R_{basal}$, where [A] is the concentration of agonist, n is the Hill coefficient and R is the response. Data are given as a mean ± S.E.M. of at least three individual experiments performed in triplicate (or in a few cases, in duplicate).

3. Results

As reported previously, we were able to obtain a robust agonist-induced response (between 3- and 5-fold), when the wild type $GABA_B$1b receptor was co-expressed with the $GABA_B$2 receptor and the chimeric G-protein Gαq-z5 (Bräuner-Osborne and Krogsgaard-Larsen, 1999). Both GABA and (*R*)-baclofen were full agonists with potencies in the low micromolar range. The pharmacological parameters of both agonists on the wild type receptor were in excellent agreement with those previously reported (Table 1) (Bräuner-Osborne and Krogsgaard-Larsen, 1999). In agreement with a recent study using [^{3}H]GABA binding to rat brain synaptic membranes and electrically induced contractions of guinea pig ileum (Karla et al., 1999), (*R*)-homobaclofen was approximately 20-fold less potent than (*R*)-baclofen. Agonist responses of 20 μM (*R*)-baclofen or 200 μM (*R*)-homobaclofen were antagonized by 100 μM *P*-(3-aminopropyl)-*P*-(diethoxymethyl)phosphonic acid (CGP35348), and (*S*)-homobaclofen was inactive as an agonist when tested at 4 mM concentration, in agreement with the results of binding studies on using rat brain homogenate (Karla et al., 1999) (data not shown).

When the Ser^{130} residue in the $GABA_B$1b receptor was mutated to an alanine (mutant S130A), the abilities of GABA, (*R*)-baclofen and (*R*)-homobaclofen to activate the receptor were dramatically impaired (Table 1). The concentration–response curve for GABA on the mutant started at concentrations above 1 mM, whereas no response was detected for either (*R*)-baclofen or (*R*)-homobaclofen in concentrations up to 3 mM.

The pharmacological profiles of GABA, (*R*)-baclofen and (*R*)-homobaclofen on the wild type and S153A $GABA_B$1b receptors are depicted in Fig. 1. Mutation of Ser^{153} resulted in a 30-fold lower potency of GABA, whereas the potency of (*R*)-baclofen was not significantly changed (Table 1). Mimicking the insignificant effect of

Table 1
EC_{50} values and Hill coefficients (n_H) from agonist-induced IP accumulation in tsA cells transfected with WT or mutant $GABA_B$1b receptors together with the $GABA_B$2 receptor and Gαq-z5. The assay was performed as described in Section 2, and the data are given as mean ± S.E.M. of three to seven independent experiments

	GABA		(*R*)-baclofen		(*R*)-homobaclofen	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
WT	1.4 ± 0.47	1.1 ± 0.22	1.7 ± 0.16	1.4 ± 0.22	36 ± 10	1.1 ± 0.17
S130A	> 3.000[a]	–[b]	> 3.000	–[b]	> 3.000	–[b]
S153A	46 ± 8.5	0.99 ± 0.19	4.3 ± 1.4	0.84 ± 0.063	53 ± 26	1.1 ± 0.23

[a] EC_{50} between 3 and 10 mM.
[b] Not determinable.

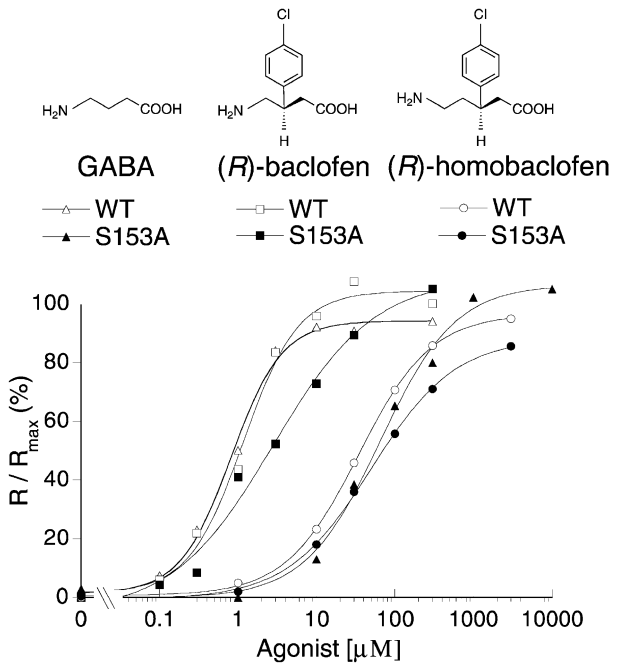

Fig. 1. Structures of GABA, (*R*)-baclofen and (*S*)-homobaclofen and concentration–response curves of the three agonists on wild type and S153A $GABA_B$R1b co-expressed with $GABA_B$R2 and the chimeric G-protein Gαq-z5. The curves are representative for the average pharmacological profile of the agonists. The inositol phosphate assays were performed as described in Section 2, and data are expressed as percentage of the maximal response of (*R*)-baclofen on the wild type receptor. Error bars are omitted for reasons of clarity.

the S153A mutation on the pharmacology of (*R*)-baclofen, (*R*)-homobaclofen retained its potencies at the wild type receptor on the mutant (Table 1 and Fig. 1).

4. Discussion

In a recent study, we showed that the two enantiomers of homobaclofen displayed atypical pharmacological profiles in comparison with the two enantiomers of baclofen (Karla et al., 1999). In $GABA_B$ receptor selective [^{3}H]GABA binding to rat brain synaptic membranes, (*R*)-homobaclofen was 50-fold less potent than (*R*)-baclofen, whereas both (*S*)-enantiomers were inactive. When tested in an assay of electrically induced contractions of guinea pig ileum, (*R*)-homobaclofen was still less potent than (*R*)-baclofen (13-fold) but interestingly, (*S*)-homobaclofen was shown to be almost equipotent with the (*R*)-enantiomer. Furthermore, in contrast to (*R*)-baclofen, agonist responses of neither (*R*)- nor (*S*)-homobaclofen could be antagonized by the classical $GABA_B$ receptor antagonist CGP35348 (Karla et al., 1999). This atypical $GABA_B$ receptor pharmacology was explained as either a receptor interaction of (*R*)- and (*S*)-homobaclofen different from that of baclofen or differences in receptor subtype selectivity of these two enantiomers (Karla et al., 1999).

Using recombinant $GABA_B$ receptors expressed in tsA cells, we also observed that (*R*)-homobaclofen acts very similar to (*R*)-baclofen, albeit with lower potency, on the wild type $GABA_B$1b receptor. However, in contrast to the previous functional study, (*R*)-homobaclofen could be antagonized by CGP35348, and (*S*)-homobaclofen displayed no effect on the $GABA_B$1b receptor.

In order to investigate the pharmacology of (*R*)-homobaclofen and (*R*)-baclofen in further detail, we mutated the Ser^{130} and Ser^{153} residues in the $GABA_B$1b receptor. Mutation of the serine residue in the $GABA_B$1a receptor corresponding to Ser^{130} in the $GABA_B$1b, Ser^{246}, has been shown to knock out binding of the competitive $GABA_B$ receptor antagonist CGP64213 and to make the receptor insensitive to agonist activation (Galvez et al., 1999, 2000a). The serine residue is believed to be involved in the binding of the carboxylate group of the agonist molecules. Furthermore, Galvez et al. (2000b) have previously demonstrated that the serine residue in $GABA_B$R1a corresponding to Ser^{153} in $GABA_B$R1b is involved in the calcium sensing of the receptor, and it was shown that this Ca^{2+} sensing ability was important for GABA but not for baclofen activation of the receptor. In agreement with these studies, we find that mutation of Ser^{130} in the $GABA_B$1b receptor impairs the ability of both GABA and (*R*)-baclofen to activate the receptor. An alanine mutation of Ser^{153} leads to a decreased potency of GABA, whereas the EC_{50} of (*R*)-baclofen is unaltered as compared to the wild type receptor (Table 1). The changes in the pharmacological profile of (*R*)-homobaclofen as a result of the two mutations closely resemble those displayed by (*R*)-baclofen, indicating that both compounds bind to Ser^{130} and that the effects of both compounds do not seem to be associated with the "Ca^{2+} sensing" properties of the receptor.

The atypical behavior of (*R*)- and (*S*)-homobaclofen on electrically induced contractions of guinea pig ileum (Karla et al., 1999) thus appear to be caused by differences in subtype selectivity or could suggest the existence of additional receptor subtypes. In recent studies, no measurable to very low levels of $GABA_B$2 receptor splice variant expression have been detected in human and rat peripheral tissues, including intestine tissue (Clark et al., 2000; Calver et al., 2000). Hence, the observed discrepancies in the pharmacology of the enantiomers of homobaclofen in guinea pig ileum could be speculated to arise from interactions of the compounds with other $GABA_B$ receptor complexes than the presumed $GABA_B1/GABA_B2$ receptor heterodimer. Although significant pharmacological differences between $GABA_B$1 and $GABA_B$2 receptor subunit splice variants have not been reported yet, it cannot be ruled out that the enantiomers of homobaclofen are capable of discriminating between such splice variants. Finally, as a third possibility it should be noted that the receptors used in this study are cloned from rat and our results correlate nicely with the previously described $GABA_B$ receptor selective [^{3}H]GABA binding to rat brain synaptic

membranes. It is thus possible that differences in amino acid sequence between rat and guinea pig $GABA_B$ receptors are the cause of the differences in pharmacology. Further experiments are needed to address these aspects.

Acknowledgements

The authors wish to thank Dr. Janet Clark for the $GABA_B$R1b-pCDNA3.1 and $GABA_B$R2-pCDNA3.1 constructs, Dr. Bruce Conklin for Gαq-z5-pCDNA3.1, and Dr. Penelope S.V. Jones and Dr. Wolfgang Fröstl for their kind gifts of the tsA cells and CGP35348, respectively. This work was supported by the Danish Medical Research Council and the Novo Nordisk Foundation.

References

Bräuner-Osborne, H., Krogsgaard-Larsen, P., 1999. Functional pharmacology of cloned heterodimeric $GABA_B$ receptors expressed in mammalian cells. Br. J. Pharmacol. 128, 1370–1374.

Bräuner-Osborne, H., Krogsgaard-Larsen, P., 2000. Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. Genomics 65, 121–128.

Bräuner-Osborne, H., Jensen, A.A., Krogsgaard-Larsen, P., 1999. Interaction of CPCCOEt with a chimeric $mGlu_{1b}$ and calcium sensing receptor. NeuroReport 10, 3923–3925.

Bräuner-Osborne, H., Egebjerg, J., Nielsen, E.Ø., Madsen, U., Krogsgaard-Larsen, P., 2000. Ligands for glutamate receptors: design and therapeutic prospects. J. Med. Chem. 43, 2609–2645.

Brown, E.M., 1999. Physiology and pathophysiology of the extracellular calcium-sensing receptor. Am. J. Med. 106, 238–253.

Calver, A.R., Medhurst, A.D., Robbins, M.J., Charles, K.J., Evans, M.L., Harrison, D.C., Stammers, M., Hughes, S.A., Hervieu, G., Couve, A., Moss, S.J., Middlemiss, D.N., Pangalos, M.N., 2000. The expression of $GABA_B1$ and $GABA_B2$ receptor subunits in the CNS differs from that in peripheral tissues. Neuroscience 100, 155–170.

Chahine, M., Bennett, P.B., George Jr., A.L., Horn, R., 1994. Functional expression and properties of the human skeletal muscle sodium channel. Pfluegers Arch. 427, 136–142.

Cheng, V., Lotan, R., 1998. Molecular cloning and characterization of a novel retenoic acid-inducible gene that encodes a putative G protein-coupled receptor. J. Biol. Chem. 273, 35008–35015.

Clark, J.A., Mezey, E., Lam, A.S., Bonner, T.I., 2000. Distribution of the $GABA_B$ receptor subunit gb2 in rat CNS. Brain Res. 860, 41–52.

Galvez, T., Parmentier, M.-L., Joly, C., Malitschek, B., Kaupmann, K., Kuhn, R., Bittiger, H., Froestl, W., Bettler, B., Pin, J.-P., 1999. Mutagenesis and modeling of the $GABA_B$ receptor extracellular domain support a venus flytrap mechanism for ligand binding. J. Biol. Chem. 274, 13362–13369.

Galvez, T., Prézeau, L., Milioti, G., Franek, M., Joly, C., Forestl, W., Bettler, B., Bertrand, H.-O., Blahos, J., Pin, J.-P., 2000a. Mapping the agonist binding site of $GABA_B$ type 1 subunit sheds light on the activation process of $GABA_B$ receptors. J. Biol. Chem. 275, 41166–41174.

Galvez, T., Urwyler, S., Prézeau, L., Mosbacher, J., Joly, C., Malitschek, B., Heid, J., Brabet, I., Froestl, W., Bettler, B., Kaupmann, K., Pin, J.-P., 2000b. Ca^{2+} requirement for high-affinity g-aminobutyric acid (GABA) binding at $GABA_B$R1 receptors: involvement of serine 269 of the $GABA_B$R1 subunit. Mol. Pharmacol. 57, 419–426.

Karla, R., Ebert, B., Thorkildsen, C., Herdeis, C., Johansen, T.N., Nielsen, B., Krogsgaard-Larsen, P., 1999. Synthesis and pharmacology of the baclofen homologues 5-amino-4-(4-chlorophenyl)pentanoic acid and the *R*- and *S*-enantiomers of 5-amino-3-(4-chlorophenyl)pentanoic acid. J. Med. Chem. 42, 2053–2059.

Möhler, H., Fritschy, J.-M., 1999. $GABA_B$ receptors make it to the top —as dimers. Trends Pharmacol. Sci. 20, 87–89.

Nanevicz, T., Wang, L., Chen, M., Ishii, M., Coughlin, S.R., 1996. Thrombin receptor activating mutations. J. Biol. Chem. 271, 702–706.

Robbins, M.J., Michalovich, D., Hill, J., Calver, A.R., Medhurst, A.D., Gloger, I., Sims, M., Middlemiss, D.N., Pangalos, M.N., 2000. Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C). Genomics 67, 8–18.

Tirindelli, R., Mucignat-Caretta, C., Ryba, N.J., 1999. Molecular aspects of pheromonal communication via the vomeronasal organ of mammals. Trends Neurosci. 21, 482–486.