

Ca²⁺ Requirement for High-Affinity γ -Aminobutyric Acid (GABA) Binding at GABA_B Receptors: Involvement of Serine 269 of the GABA_BR1 Subunit

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ABSTRACT

The γ -aminobutyric acid (GABA) receptor type B (GABA_BR) is constituted of at least two homologous proteins, GABA_BR1 and GABA_BR2. These proteins share sequence and structural similarity with metabotropic glutamate and Ca²⁺-sensing receptors, both of which are sensitive to Ca²⁺. Using rat brain membranes, we report here that the affinity of GABA and 3-aminopropylphosphinic acid for the GABA_BR receptor is decreased by a factor >10 in the absence of Ca²⁺. Such a large effect of Ca²⁺ is not observed with baclofen or the antagonists CGP64213 and CGP56999A. In contrast to baclofen, the potency of GABA in stimulating GTP γ S binding in rat brain membranes is also decreased by a factor >10 upon Ca²⁺ removal. The potency for Ca²⁺ in regulating GABA affinity was 37 μ M. In cells expressing GABA_BR1, the potency of GABA, but not of

baclofen, in displacing bound ¹²⁵I-CGP64213 was similarly decreased in the absence of Ca²⁺. To identify residues that are responsible for the Ca²⁺ effect, the pharmacological profile and the Ca²⁺ sensitivity of a series of GABA_BR1 mutants were examined. The mutation of Ser269 into Ala was found to decrease the affinity of GABA, but not of baclofen, and the GABA affinity was found not to be affected upon Ca²⁺ removal. Finally, the effect of Ca²⁺ on the GABA_B receptor function is no longer observed in cells coexpressing this GABA_BR1-S269A mutant and the wild-type GABA_BR2. Taken together, these results show that Ser269, which is conserved in the GABA_BR1 protein from *Caenorhabditis elegans* to mammals, is critical for the Ca²⁺-effect on the heteromeric GABA_B receptor.

The neurotransmitter γ -aminobutyric acid (GABA) activates both ionotropic receptors (the GABA_A and GABA_C receptors) and G protein-coupled metabotropic receptors (the GABA_B receptors). GABA_A and GABA_C receptors are permeable to Cl[−] ions and mediate, in most cases, fast inhibition of the postsynaptic neurons. GABA_B receptors mostly couple to pertussis toxin-sensitive G proteins (Gi or Go) and accordingly inhibit the activity of adenylyl cyclase and voltage-gated Ca²⁺-channels or activate G protein-regulated inwardly rectifying K⁺-channels (Kir3) (for reviews, see Kerr and Ong, 1995; Bettler et al., 1998). When located on the postsynaptic element, the GABA_B receptors inhibit neuronal excitability (Kerr and Ong, 1995; Bettler et al., 1998). GABA_B

receptors are also found on glutamatergic or GABA-ergic terminals, where they inhibit neurotransmitter release. GABA_B receptors are widely expressed in the central nervous system, both in brain and spinal cord. Accordingly, these receptors play important physiological roles in pain transmission, absence epilepsy, and drug addiction (Kerr and Ong, 1995; Bettler et al., 1998).

Two GABA_B receptor proteins have been identified: GABA_BR1 (Kaupmann et al., 1997) and GABA_BR2 (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999; Ng et al., 1999). Neither receptor was found to efficiently couple to G proteins when expressed alone (Kaupmann et al., 1997, 1998a,b; Martin et al., 1999). Indeed, GABA_B receptors that efficiently activate pertussis-toxin-sensitive G proteins were only obtained in cells coexpressing GABA_BR1 and GABA_BR2 (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999; Ng et al.,

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ABBREVIATIONS. GABA, γ -aminobutyric acid; GPCR, G protein-coupled receptor; mGluR, metabotropic glutamate receptor; CaSR, Ca²⁺-sensing receptor; HEK, human embryonic kidney; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); IP, inositol phosphates; APPA, 3-aminopropylphosphinic acid; LBP, leucine binding protein; PLC, phospholipase C; LIVBP, leucine/isoleucine/valine binding protein.

1999). Moreover, these two receptors were found to associate at least by their carboxyl-terminal sequence (White et al., 1998; Kuner et al., 1999), further suggesting that GABA_B receptors function in a heteromeric form. Both GABA_BR1 and R2 proteins share sequence similarity with family 3 G protein-coupled receptors (GPCRs) (Bockaert and Pin, 1999), which include metabotropic glutamate receptors (mGluRs), Ca²⁺-sensing receptors (CaSR) and putative pheromone and taste receptors. Like all family 3 GPCRs, GABA_B receptors possess a large amino-terminal domain structurally related to some bacterial periplasmic proteins, where ligands bind (Galvez et al., 1999; Malitschek et al., 1999). This amino-terminal domain is attached to a heptahelical transmembrane domain that constitutes the G protein-activating domain, as demonstrated for mGluRs (Pin et al., 1994; Gomeza et al., 1996a).

Because GABA_B receptors share sequence similarity with mGluRs and CaSRs, two types of receptors sensitive to Ca²⁺ ions (Brown and Hebert, 1997; Kubo et al., 1998; Saunders et al., 1998), we examined a possible regulation of GABA_B receptors by Ca²⁺ ions. We found that Ca²⁺ affects the affinity of various GABA_B ligands differently and identified an amino acid residue in GABA_BR1 that is critical for this effect.

Experimental Procedures

Materials. GABA was obtained from Sigma (L'Isle d'Abeau, France). [¹²⁵I]-CGP64213 was synthesized from ethyl-(1,1-diethoxyethyl)phosphonate as described elsewhere (Froestl et al., 1995) and labeled to a specific radioactivity of >2000 Ci/mmol (ANAWA AG, Wangen, Switzerland). All the other ligands were synthesized in-house (Froestl et al., 1995). Serum, culture media, and other solutions used for cell culture were from Life Technologies, Inc. (Cergy Pontoise, France).

Culture and Transfection of 293 Cells. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Cergy Pontoise, France) supplemented with 10% fetal calf serum and transfected by electroporation as described previously (Gomeza et al., 1996b). Electroporation was carried out in a total volume of 300 μ l with 10 μ g of carrier DNA, plasmid DNA containing the wild-type or mutated GABA_BR1 (1 μ g) (Galvez et al., 1999), GABA_BR2 (1 μ g) (Kaupmann et al., 1998a), or Gqi9 (1 μ g) (Conklin et al., 1993) and 10 million cells.

Ligand Binding Assay. Rat brain membranes were prepared as described previously (Olpe et al., 1990) and stored at -70°C. Membranes of HEK 293 cells were prepared as follows: 24 h after transfection, the cells were transferred into serum-free Dulbecco's modified Eagle's medium. Forty-eight hours after transfection, the cells were washed and homogenized in Tris-Krebs buffer [20 mM Tris-Cl, pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 4.7 mM KCl, 1.8 mM CaCl₂ (omitted for calcium-free experiments)] and centrifuged for 20 min at 40,000g. The pellet was resuspended in Tris-Krebs buffer and stored at -80°C. For ligand competition assays, thawed membranes (10 μ g of protein) were incubated with 0.1 nM [¹²⁵I]-CGP64213 in the absence or presence of unlabeled ligands at different concentrations, as indicated on the figures. This concentration of [¹²⁵I]-CGP64213 is about 10 times lower than the affinity of this radioligand on the native or GABA_BR1 receptor, such that the IC₅₀ values measured for all unlabeled compounds are not significantly different from their affinity. The non-specific binding was determined using 10 mM GABA or 100 nM CGP54626A. The incubation was terminated by filtration through GF/C Whatman glass fiber filters (Whatman International Ltd., Maidstone, England).

GTP γ S Assay. Rat brain synaptic membranes were prepared as described previously (Olpe et al., 1990) and stored in -70°C. To

perform this assay on recombinant receptors, membranes were prepared from Chinese hamster ovary K1 cells (American Type Culture Collection, Manassas, VA) stably transfected with GABA_BR1b and GABA_BR2 cDNAs. Human GABA_BR1b and rat GABA_BR2 constructs [R1 in pcDNA3.1, Invitrogen (San Diego, CA); R2 in pC1-neo (Promega, Madison, WI)] were cotransfected (1:1 ratio of plasmids) using the Superfect transfection system from Qiagen AG (Basel, Switzerland). Stably transfected cell clones were selected by double selection with 1 mg/ml geneticin and 250 μ g/ml zeocin. Membranes were thawed using an ice-water bath and centrifuged at 4°C for 15 min at 20,000g. After addition of 1.2 ml of ice-cold water, the pellet was shaken, incubated on ice for 1 h, and centrifuged again. Afterward, the pellet was homogenized in guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) buffer (50 mM Tris-Cl, pH 7.7, 10 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl) using a glass/Teflon homogenizer. Assay mixtures including membranes (20–30 μ g) and test substances were prepared in Packard-Pico-Plates 96 (300- μ l volume), supplemented with 30 μ M GDP (Sigma) (10 μ M in experiments conducted with recombinant receptors) and incubated for 30 min at room temperature. When experiments were conducted in the presence of Ca²⁺, CaCl₂ was added at a concentration of 2.0 mM (final free Ca²⁺ concentration, 1.8 mM). The main incubation started by the addition of [³⁵S]GTP γ S (Amersham Paisley, UK) to a final concentration of 0.2 nM (0.3 nM in experiments conducted with recombinant receptors). Nonspecific binding was measured in the presence of 10 μ M unlabeled GTP γ S. After 40 min incubation at room temperature, the amount of bound [³⁵S]GTP γ S was assessed by standard procedures such as vacuum filtration through GF/B filters followed by liquid scintillation counting or scintillation proximity assay using 1.5 mg of wheat germ agglutinin-coated scintillation proximity assay beads (Amersham) per 250 μ l of assay mixture.

Determination of Inositol Phosphate Accumulation. Determination of inositol phosphate (IP) accumulation in transfected cells was performed after labeling the cells overnight with myo-[³H]inositol (23.4 Ci/mol; NEN, Les Ullis, France). The stimulation was conducted for 30 min in a medium containing 10 mM LiCl and the indicated concentration of agonist. Results are expressed as the amount of IP produced over the radioactivity present in the membranes.

Data Analysis. The curves were fitted with either GraphPAD Prism software (GraphPAD Software, San Diego, CA) or Kaleidagraph software (Abelbeck/Synergy Software, Reading, PA) using the equation $y = (y_{\max} - y_{\min}) / [1 + (x/EC_{50})^{n_H}] + y_{\min}$, where EC₅₀ is the concentration of the compound necessary to obtain 50% of the maximal effect and n_H is the Hill coefficient.

Results

Ca²⁺ Differently Regulates the Affinity of Several Compounds on Native GABA_B Receptors. The effect of Ca²⁺ removal on the affinity of several ligands interacting with the GABA_B receptor was examined in rat brain membranes using [¹²⁵I]-CGP64213 as radioligand. This compound has previously been reported to selectively label GABA_BR1 proteins in the mammalian brain (Kaupmann et al., 1997). As shown in Table 1, the potencies of cold CGP64213 and of CGP56999A, another antagonist, were only slightly affected upon removal of Ca²⁺ (1 mM EGTA added). A more pronounced effect of Ca²⁺ was, however, observed with the other antagonist CGP54626A. In contrast, the potency of the agonists GABA and APPA were decreased by a factor of more than 10 (Table 1). This effect is not a general effect for all GABA_BR agonists because the potency of baclofen was not affected by Ca²⁺ removal (Table 1). This indicates that Ca²⁺ is necessary for high-affinity binding of some, but not all, compounds interacting with native GABA_BR1. Because of the

TABLE 1

Effect of Ca²⁺ on the affinities of several GABA_B receptor ligands on native GABA_B receptors

[¹²⁵I]CGP64213 binding on rat brain membranes (native receptors) or on membranes prepared from HEK 293 cells expressing GABA_BR1a receptors (GABA_BR1a) was measured in the absence and presence of increasing concentrations of the indicated compounds. IC₅₀ values (micromolar for the agonists GABA, APPA and baclofen, and nanomolar for the antagonists CGP64213, CGP54626A and CGP56999A) were determined as described under *Experimental Procedures*. Experiments were conducted in the presence of 1.8 mM Ca²⁺ (Ca²⁺), or in the presence of 1 mM EGTA with no added Ca²⁺ (EGTA). Values are means \pm S.E.M. of at least three independent experiments performed in triplicate. The ratio of the mean IC₅₀ value measured in the absence of Ca²⁺ over that measured in the presence of Ca²⁺ is also indicated for each compound (EGTA/Ca²⁺) for both native and recombinant GABA_BR1a receptors.

	Native receptors			GABA _B R1a		
	Ca ²⁺	EGTA	EGTA/Ca ²⁺	Ca ²⁺	EGTA	EGTA/Ca ²⁺
Agonists	IC ₅₀ (μ M)	IC ₅₀ (μ M)	IC ₅₀ /IC ₅₀	IC ₅₀ (μ M)	IC ₅₀ (μ M)	IC ₅₀ /IC ₅₀
GABA	0.27 \pm 0.02	6.8 \pm 0.36	25.2	20.9 \pm 0.8	780 \pm 143	37.3
APPA	0.036 \pm 0.004	0.47 \pm 0.10	13.0	2.8 \pm 0.6	149 \pm 20	53.2
Baclofen	0.45 \pm 0.09	0.45 \pm 0.02	1.0	30.2 \pm 1.7	34.2 \pm 7.2	1.13
Antagonists	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ /IC ₅₀	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ /IC ₅₀
CGP64213	1.17 \pm 0.02	2.00 \pm 0.20	1.7	1.53 \pm 0.25	2.74 \pm 0.96	1.79
CGP54626A	2.8 \pm 0.1	8.6 \pm 1.3	3.1	1.90 \pm 0.22	5.4 \pm 0.8	2.84
CGP56999A	0.6 \pm 0.1	0.84 \pm 0.02	1.4	0.38 \pm 0.06	0.26 \pm 0.06	0.68

low concentration of [¹²⁵I]-CGP64213 used (1/10 of its K_d value), the IC₅₀ values measured for unlabeled compounds are very near their K_i (affinity) values.

We also examined the effect of Ca²⁺ on the potency of GABA and baclofen to activate native GABA_B receptors. In the presence of 1 mM Ca²⁺, GABA, APPA, and baclofen stimulated GTP γ S binding in a dose-dependent manner (Table 2). In the absence of Ca²⁺, the EC₅₀ values for GABA and APPA were increased by a factor 80 whereas that of baclofen was only slightly affected (Fig. 1). When the Log(EC₅₀) values for GABA determined at various Ca²⁺ concentrations were plotted against the Ca²⁺ concentration, an EC₅₀ value of 37 \pm 5 μ M was determined for Ca²⁺ (Fig. 1a, inset).

Effect of Ca²⁺ on the Recombinant GABA_B Receptors. Because [¹²⁵I]-CGP64213 has been reported to bind on the GABA_BR1 subunit but not on the GABA_BR2 subunit (Kaupmann et al., 1998a), we first examined the effect of Ca²⁺ on the recombinant GABA_BR1a subunit expressed alone in HEK 293 cells. As shown in Table 1, the affinities of CGP64213, CGP56999A, and baclofen were not affected by removal of Ca²⁺, but those of GABA and APPA were largely decreased (Fig. 2 and Table 1). Similar data were obtained with the GABA_BR1b splice variant, which lacks the 120 amino-terminal residues that correspond to the sushi domains of GABA_BR1a (data not shown) (Kaupmann et al., 1997; Hawrot et al., 1998).

As observed with the native receptors, Ca²⁺ was also required for the high potency of GABA and APPA to stimulate [³⁵S]GTP γ S binding on membranes prepared from cells stably expressing both GABA_BR1 and GABA_BR2 subunits (Table 2). On these membranes, the effect of baclofen was found

to be unaffected by Ca²⁺ removal (Table 2). The shift in GABA and APPA potencies observed upon removal of Ca²⁺ was, however, found to be lower on the recombinant receptor than on the native receptors (Table 2).

Effect of Point Mutations in GABA_BR1 on the Ca²⁺-Effect. Because Ca²⁺ affects both the potency measured in a functional assay and the affinity of GABA and APPA, it is likely that Ca²⁺ acts directly on the ligand-binding suppress domain of the GABA_BR1 protein. We therefore first examined whether the effect of Ca²⁺ could still be observed on a truncated GABA_BR1 receptor protein that corresponds to the LBP-like domain attached to the plasma membrane by only one transmembrane segment. We previously reported that such a truncated receptor still binds [¹²⁵I]-CGP64213 (Malitschek et al., 1999). On such a truncated protein, the affinity of GABA, but not of baclofen, was also decreased in the absence of Ca²⁺ (data not shown), indicating that Ca²⁺ interacts on the LBP-like domain of the GABA_BR1 protein.

Because of the specific effect of Ca²⁺ on the affinity of some but not all GABA_B ligands, we speculated that the amino acid residues responsible for the Ca²⁺ effect are near the GABA binding site. In accordance with this idea, a single residue (Ser166) in mGluR1, located near a serine residue involved in glutamate binding (Ser165), plays a critical role in the Ca²⁺ effect on this receptor subtype (Kubo et al., 1998). These two serine residues are conserved in GABA_BR1a (Ser247 and Ser246, respectively) (Fig. 3), and using molecular modeling and mutagenesis, we showed that Ser246 is probably directly involved in the binding of GABA (Galvez et al., 1999). One would therefore predict that Ser247 could be responsible for the Ca²⁺ effect in GABA_BR1a. In addition to Ser247, at least

TABLE 2

Effect of Ca²⁺ on the potencies of several GABA_B receptor agonists in stimulating [³⁵S]GTP γ S binding on native and recombinant GABA_B receptors

[³⁵S]GTP γ S binding on rat brain membranes (native receptors) or on membranes prepared from Chinese hamster ovary K2 cells stably expressing the human GABA_BR1b and the rat GABA_BR2 was measured in the absence and presence of increasing concentrations of the indicated compounds. EC₅₀ values (micromolar) were determined as described under *Experimental Procedures*. Experiments were conducted in the presence of 1.8 mM Ca²⁺, or in the presence of 0.2 mM EGTA with no added Ca²⁺. Values are means \pm S.E.M. of three to seven independent experiments performed in triplicate. The ratio of the mean EC₅₀ value measured in the absence of Ca²⁺ over that measured in the presence of Ca²⁺ is also indicated for each compound (EGTA/Ca²⁺) for both native and recombinant GABA_B receptors.

	Native receptors			GABA _B R1b + GABA _B R2		
	Ca ²⁺	EGTA	EGTA/Ca ²⁺	Ca ²⁺	EGTA	EGTA/Ca ²⁺
Agonists	EC ₅₀ (μ M)	EC ₅₀ (μ M)	EC ₅₀ /EC ₅₀	EC ₅₀ (μ M)	EC ₅₀ (μ M)	EC ₅₀ /EC ₅₀
GABA	2.4 \pm 0.1	200 \pm 20	83.3	4.0 \pm 0.6	91 \pm 9	22.7
APPA	0.45 \pm 0.01	36 \pm 9	80.0	0.45 \pm 0.08	13 \pm 2	28.9
Baclofen	3.2 \pm 0.2	17 \pm 2	5.3	7.0 \pm 0.6	6.6 \pm 1.1	0.94

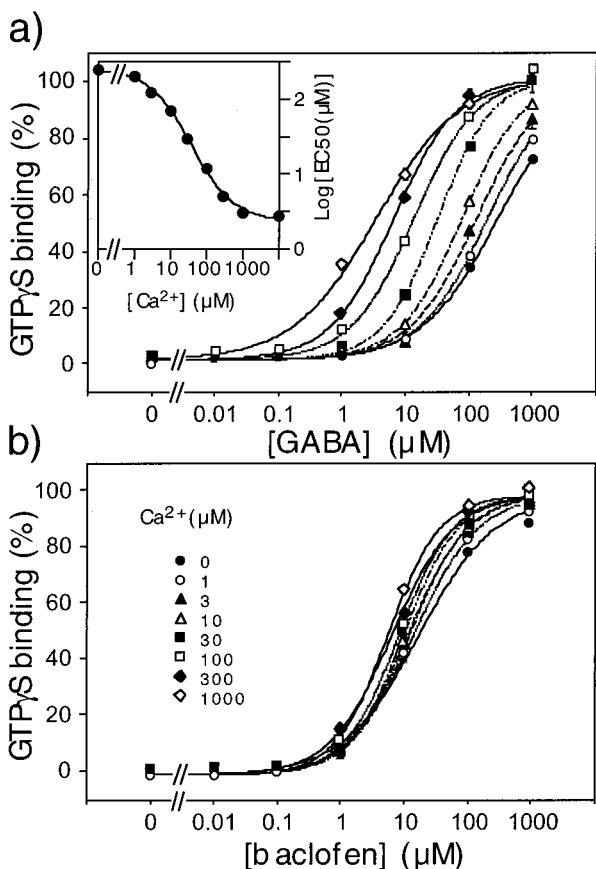


Fig. 1. Effect of Ca^{2+} on GABA (a) and baclofen (b) stimulated $\text{GTP}\gamma\text{S}$ binding on rat brain membranes. The binding of [^{35}S] $\text{GTP}\gamma\text{S}$ on rat brain membranes was measured in the presence of the indicated concentration of GABA (a) or baclofen (b), and in the presence of the indicated concentration of Ca^{2+} : 0 μM (●), 1 μM (○), 3 μM (▲), 10 μM (△), 30 μM (■), 100 μM (▀), 300 μM (◆), 1000 μM (◇). Data are means \pm S.E.M. of a triplicate determination from a typical experiment. Inset in a: plot of the EC_{50} value for GABA as a function of Ca^{2+} concentration.

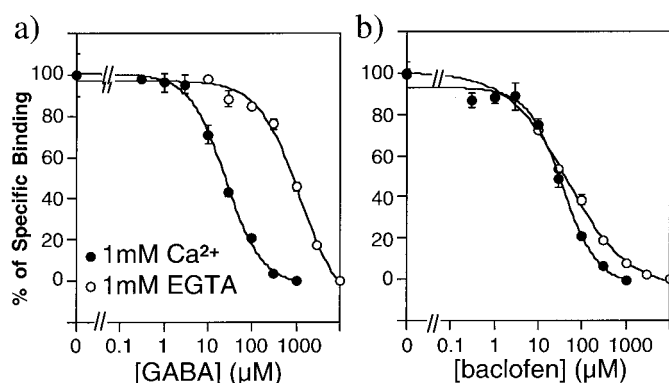


Fig. 2. Inhibition curves of [^{125}I]-CGP64213 binding on membranes prepared from cells expressing $\text{GABA}_\text{B}\text{R1a}$ by GABA and baclofen in the presence (a) and absence (b) of Ca^{2+} . The binding of 0.1 nM [^{125}I]-CGP64213 on HEK 293 cell membranes (10 μg) was measured in the presence of the indicated concentration of GABA (○) or baclofen (●), and in the presence (a) or absence (b, 1 mM EGTA added) of added Ca^{2+} (1 mM). Data are means \pm S.E.M. of triplicate determinations from a single typical experiment.

two other serine residues (Ser268 and Ser269) are also near Ser246 in our three-dimensional model of the $\text{GABA}_\text{B}\text{R1a}$ binding site. Interestingly, Ser269 of $\text{GABA}_\text{B}\text{R1a}$ aligned with a serine residue of the CaSR that plays an important

role for its activation by Ca^{2+} (Bräuner-Osborne et al., 1999) (see Fig. 3).

Therefore, the effect of the mutation of Ser247, Ser268, and Ser269 into Ala on the potency of several GABA_B ligands to displace [^{125}I]-CGP64213 specific binding was examined. The potency of CGP64213 was not significantly affected in any of these mutated receptors [$\text{IC}_{50} = 1.44 \pm 0.33$, 0.94 ± 0.15 , and 4.48 ± 0.84 nM ($n = 3$) on $\text{GABA}_\text{B}\text{R1a-S247A}$, -S268A, and -S269A mutants, respectively, compared with 1.53 ± 0.25 nM for the wild-type receptor]. Moreover, the potency of CGP64213 was affected by a factor of ~ 2 upon removal of Ca^{2+} ($\text{IC}_{50} = 3.25 \pm 0.40$, 1.98 ± 0.17 , and 7.04 ± 2.11 nM ($n = 3$) on $\text{GABA}_\text{B}\text{R1a-S247A}$, -S268A, and -S269A mutants, respectively).

If one of the above-mentioned residues is involved in the Ca^{2+} -sensitivity of $\text{GABA}_\text{B}\text{R1a}$, its mutation into an alanine is expected to have an effect similar to that resulting from Ca^{2+} removal: a large decrease in the affinity for GABA but not for baclofen. Indeed, among the three mutants, only $\text{GABA}_\text{B}\text{R1a-S269A}$ displayed these features, as reported previously (Galvez et al., 1999) (Table 3). Moreover, Ca^{2+} removal from the incubation buffer resulted in a decreased affinity for GABA in $\text{GABA}_\text{B}\text{R1a-S247A}$ and $\text{GABA}_\text{B}\text{R1a-S268A}$ mutants but not in the $\text{GABA}_\text{B}\text{R1a-S269A}$ mutant (Fig. 4 and Table 3), further indicating that Ser269 plays a critical role in the Ca^{2+} effect.

Ser269 of the $\text{GABA}_\text{B}\text{R1}$ Receptor Subunit Plays a Critical Role for the Ca^{2+} -Effect on the Heteromeric GABA_B Receptor. The effect of the mutation of Ser269 in $\text{GABA}_\text{B}\text{R1a}$ was examined on the functional activity of the heteromeric $\text{GABA}_\text{B}\text{R1}/\text{GABA}_\text{B}\text{R2}$ receptor. To that aim, $\text{GABA}_\text{B}\text{R1a}$ and $\text{GABA}_\text{B}\text{R2}$ were coexpressed in HEK 293 cells together with the chimeric G protein α -subunit Gqi9 [Gaq in which nine residues of the carboxyl-terminus have been replaced by those of G α_i (Conklin et al., 1993)]. Under these conditions, the GABA_B receptor was found to stimulate phospholipase C (PLC), and display pharmacological properties similar to those measured using other functional assays (Franeek et al., 1999). Using this assay, and as observed with other functional readouts, GABA and baclofen display a similar potency in the presence of 1 mM Ca^{2+} (Fig. 5 and Table 3). In the absence of extracellular Ca^{2+} , GABA was 10 times less potent than baclofen (Fig. 5a and Table 3), as observed with the native receptor or the recombinant heteromeric receptor using the $\text{GTP}\gamma\text{S}$ binding assay (Table 2).

However, using the PLC assay, we found that the potency of baclofen was also decreased in the absence of Ca^{2+} , although to a lower extent than that of GABA (Table 3). Because this effect was observed neither on the potency of baclofen to displace [^{125}I]-CGP64213 binding nor on its potency to stimulate [^{35}S] $\text{GTP}\gamma\text{S}$ binding (see above), it probably resulted from a Ca^{2+} requirement in a step downstream of the G protein activation by the GABA_B receptor. Indeed, Ca^{2+} is known to have a positive effect on PLC activity (Cockcroft and Thomas, 1992) such that the removal of Ca^{2+} from the external milieu can affect the coupling efficacy of a receptor to PLC, leading to a decrease in both potency and efficacy of agonists. In agreement with this proposal, the maximal effect not only of baclofen but also of GABA or APPA was decreased by 2- to 3-fold (Fig. 5A), indicating it is a general feature of the PLC cascade activation upon stimulation of the receptors by any agonist. Moreover, the effect of

Ca²⁺ on the potency of baclofen in stimulating IP formation occurred in a concentration range (30–100 μ M) different from that affecting the potency of GABA (1–30 μ M) in this assay. According to this additional effect of Ca²⁺ on the PLC activation, the effect of Ca²⁺ on the GABA potency on wild-type and mutated receptors was always compared with that of baclofen.

GABA and baclofen still activated in a dose-dependent manner the heteromeric GABA_B receptors, constituted of the wild-type GABA_BR2 and either the GABA_BR1a-S247A or GABA_BR1a-S268A mutant. These two agonists displayed a similar affinity on these two mutant receptors in the pres-

ence of Ca²⁺, whereas GABA became much less potent than baclofen in the absence of Ca²⁺ (Table 3). These data are very similar to those obtained with the wild-type receptor, further indicating that these two serine residues are not involved in the Ca²⁺-sensitivity of the GABA activation of the heteromeric receptor.

In cells expressing the GABA_BR1a-S269A mutant and GABA_BR2, we noticed a decrease by about a factor 10 of the GABA potency but no change in the EC₅₀ value for baclofen compared with the wild-type receptor (Fig. 5 and Table 3). Interestingly, this difference in the GABA potency mirrors the change in potency of GABA observed on the wild-type

	166	188
mGluR1a	IAGVIGPGSS	SVAIQVQNLLQLDFIPQIAYSATSIDLSDKT
mGluR5a	IVGVIGPGSS	SVAIQVQNLLQLNFIPQIAYSATSMDLSDKT
Cel-mGluRI	VVAVIGPGKS	SSTIAVQNLLQVRFIPQVGYSATTPDLSDKE
mGluR2	VTGVIGGSYS	DVSIQVANLLRLQFIPQISYASTSAKLSDKS
mGluR3	IAGVIGGSYS	SVSIQVANLLRLQFIPQISYASTSAKLSDKS
DmGluRA	VFGVIGGSYS	SVSLQVANLLRLHFIPQVSPASTAKTLSDKT
Cel-mGluRII	VAAVVGGSYS	SVSVQLANLLRLRFIAQVSPASTNADLSDKN
mGluR4a	VVGVIASGS	SVSIMVANILRLKFIPQISYASTAPDLSDNS
mGluR6	VVAVVGASAS	SVSIMVANVLRALAFIPQISYASTAPELSDST
mGluR7a	VVGVIASGS	SVSIMVANILRLQFIPQISYASTAPELSDDR
mGluR8a	ISGVIGAAAS	SVSIMVANILRLKFIPQISYASTAPELSDNT
	170	
BoPCaR1	TIAVVGATGSG	ISTAVANLLGLYFIPQVSYASSRLLSNKN
	246	269
rat-BR1	IKIILMPGCS	SVSTLVAAEARMWNILIVLSYGS
dro-BR1	QKLMLLAGCST	VCTTVAEAAKWNILIVVSYGASSPALSNDK
Cel-BR1	TKLMLLTGCS	PVTTVIAEAAAPVWKLVLVLSYGGSSPALSNDK
rat-BR2	HLMVFGGVCPS	VTSTIAESLQGNLVLQLSFAATTPVLADKK
dro-BR2	MVMLLGSACSE	VTESLAKVVPYWNIVQVSFGSTSPALSDDR
Cel-BR2	PVAAILGGQCTE	VSEPIAMALKYWQIVQLSYAETHAKFASDD
	79	102
LIVBP	<u>IKYVIGHLCSSSTQ</u>	<u>PASDIYEDGEILMITPAATAPELTARG</u>
LBP	<u>IKYVIGHLCSSSTQ</u>	<u>PASDIYEDGEILMISPGATAPELTORG</u>

Fig. 3. Alignment of the mGlu, Ca-sensing and GABA_B receptor sequences with those of the periplasmic binding proteins LIVBP and LBP, in one of their regions responsible for ligand binding. Residues highlighted in gray have been shown (for LIVBP and LBP) or proposed to directly interact with the ligand (Glu in mGluRs, GABA in GABA_BR1, and leucine in LIVBP). Residues highlighted in black are responsible for the effect of Ca²⁺ in mGluRs and CaSR, and that we identified as critical for the effect of Ca²⁺ on GABA_BR1. The numbers on top of the sequences correspond to the position of the residues in the rat mGluR1, bovine CaSR, rat GABA_BR1a (all including the signal peptide sequence) and LIVBP (from top to bottom). The known secondary structural elements of LIVBP and LBP are underlined (β strands in italic, and α helices in bold). The sequences of the *D. melanogaster* (dro) GABA_BR1 and GABA_BR2 were obtained from cosmid DS00929 and DS02649, respectively. The *C. elegans* (Cel) sequences Cel-BR1 and Cel-BR2 were obtained from cosmid Y41G9 and ZK180, respectively. The *C. elegans* sequences Cel-mGluRI and Cel-mGluRII were obtained from the cosmid F45H11 and ZC506, respectively. The alignment was generated using the sequence of the LBP-like domain of the receptors with the default parameters of the Clustal-W program.

TABLE 3

Effect of Ca²⁺ on the affinities and potencies of GABA and baclofen on wild-type and mutant GABA_BR1a receptors

[¹²⁵I]CGP64213 binding on membranes prepared from HEK 293 cells expressing the indicated GABA_BR1 receptor was measured in the absence and presence of increasing concentration of the indicated compounds. IC₅₀ values (micromolar) were determined as described under *Experimental Procedures*. The potencies of GABA and baclofen in activating the wild-type and mutants GABA_B receptors were determined in HEK 293 cells coexpressing the wild-type and mutant GABA_BR1, the wild-type GABA_BR2, and the chimeric G protein Gq19. Experiments were conducted in the presence of Ca²⁺, or in the presence of 1 mM EGTA with no added Ca²⁺ (binding experiments), or 5 mM EGTA and 0.1 mM Ca²⁺ (IP experiments). Values are means \pm S.E.M. of at least three independent experiments performed in triplicate. In the last two columns, the ratios GABA/baclofen EC₅₀ values measured in the presence (Ca²⁺) or absence (EGTA) of Ca²⁺ are presented.

	Ca ²⁺ [EC ₅₀ (μM)]		EGTA [EC ₅₀ (μM)]		Ca ²⁺ Ratio	EGTA Ratio
	GABA	Baclofen	GABA	Baclofen		
^[125] I]CGP64213 binding						
WT	20.9 ± 0.8	30.2 ± 1.7	780 ± 143	34.2 ± 7.2	0.69	22.8
S247A	5.32 ± 0.44	11.3 ± 0.9	287 ± 45	14.9 ± 11.5	0.42	19.3
S268A	27.8 ± 1.9	43.0 ± 4.1	327 ± 51	28.7 ± 4.7	0.64	11.4
S269A	238 ± 28	28.8 ± 5.4	227 ± 55	31.2 ± 0.9	8.26	7.27
IP formation						
WT	2.95 ± 0.20	3.15 ± 0.44	179 ± 9	22.9 ± 2.1	0.93	7.81
S247A	0.44 ± 0.04	0.38 ± 0.04	32.7 ± 11	2.57 ± 0.08	1.16	12.7
S268A	1.02 ± 0.15	0.91 ± 0.41	47.2 ± 0.2	6.22 ± 3.77	1.12	7.58
S269A	14.9 ± 0.8	1.78 ± 0.22	212 ± 13	21.2 ± 1.5	8.37	10.0

WT, wild-type.

receptor upon Ca^{2+} removal. In addition, the relative potency of GABA over that of baclofen on cells expressing $\text{GABA}_B\text{R1a-S269A}$ and $\text{GABA}_B\text{R2}$ remained unchanged in the absence of external Ca^{2+} (Fig. 5b and Table 3). This shows that the $\text{GABA}_B\text{R1a-S269A}/\text{GABA}_B\text{R2}$ heteromer is no longer sensitive to Ca^{2+} .

In brain membranes, we noticed that the removal of Ca^{2+} largely decreased not only the affinity of GABA, but also that of APPA. If Ser269 is playing a critical role for the Ca^{2+} effect on the GABA_B receptor, not only the affinity of GABA, but also that of APPA should be affected by this mutation. As shown in Table 4, this is exactly what we observed. Moreover, this mutation largely decreased the effect of Ca^{2+} on the affinity of APPA (Table 4). Surprisingly, whereas the affinity of CGP54626A and CGP56999A on either wild-type or recombinant receptors is slightly decreased upon Ca^{2+} removal (Table 1), the mutation of Ser269 into alanine resulted in a large decrease in the affinity of CGP54626A but not of CGP56999A (Table 4). This revealed that the effect of the mutation of Ser269 on the affinity of CGP54626A cannot be

simply explained by the suppression of the action of Ca^{2+} on the $\text{GABA}_B\text{R1}$ subunit.

Discussion

The present data show that Ca^{2+} is necessary for the high affinity of GABA and APPA on native and recombinant GABA_B receptors. Surprisingly, the action of baclofen on this receptor does not require Ca^{2+} ions. Such a specific decrease in GABA and APPA affinities on wild-type receptor observed upon Ca^{2+} removal was mimicked by mutating the Ser269 of $\text{GABA}_B\text{R1a}$ into Ala. Moreover, Ca^{2+} removal has no further

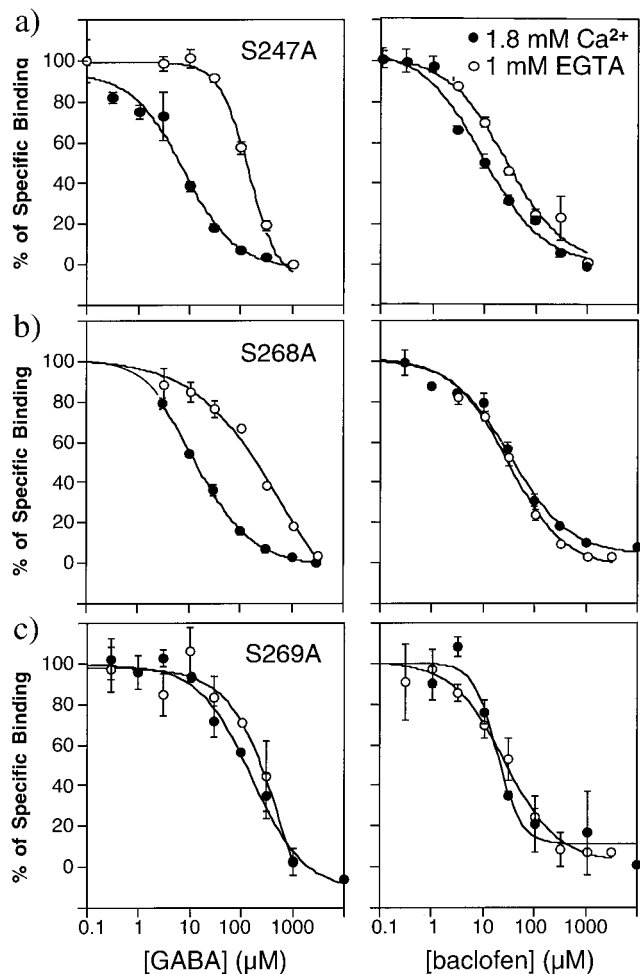


Fig. 4. Ca^{2+} -sensitivity of GABA binding on S247A (a), S268A (b), and S269A (c) $\text{GABA}_B\text{R1a}$ mutants. The binding of 0.1 nM ^{125}I -CGP64213 on membranes prepared from HEK 293 cells expressing the indicated receptor was measured in the presence of the indicated concentration of GABA (○) or baclofen (●), and in the presence (left) or absence (plus 1 mM EGTA) of added Ca^{2+} (1 mM). Data are means \pm S.E.M. of a triplicate determination from a typical experiment. For each mutant, the data with GABA and baclofen with and without Ca^{2+} were obtained in the same experiment.

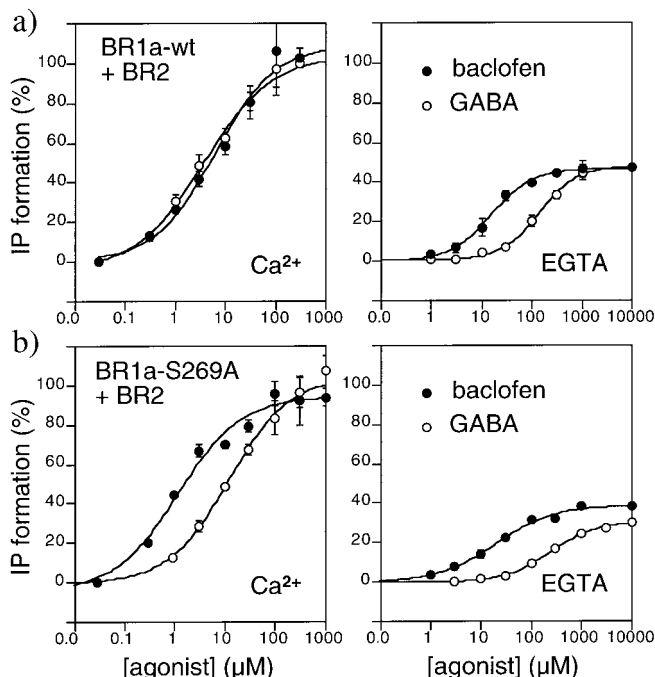


Fig. 5. Effect of Ca^{2+} on GABA and baclofen induced activation of wild-type or $\text{GABA}_B\text{R1a-S269A} + \text{GABA}_B\text{R2}$ receptors. The IP formation induced by various concentrations of GABA (○) or baclofen (●) was measured on HEK 293 cells expressing the chimeric G protein Gq19, $\text{GABA}_B\text{R2}$, and either the wild-type $\text{GABA}_B\text{R1a}$ (a) or the $\text{GABA}_B\text{R1a-S269A}$ mutant (b). The experiment was conducted either in the presence (left) or absence (plus 1 mM EGTA) of added Ca^{2+} (1 mM). Data are means \pm S.E.M. of a triplicate determination from a typical experiment. For each mutant, the data obtained with GABA and baclofen, with and without Ca^{2+} were obtained in the same experiment.

TABLE 4

Effect of Ca^{2+} on the affinities of several compounds on the $\text{GABA}_B\text{R1a-S269A}$ mutant compared with the wild-type receptor

^{125}I -CGP64213 binding on membranes prepared from HEK 293 cells expressing the indicated receptor was measured in the absence and presence of increasing concentrations of the indicated compounds. IC_{50} values were determined as described under *Experimental Procedures*. Experiments were conducted in the presence of 1 mM Ca^{2+} , or in the presence of 1 mM EGTA with no added Ca^{2+} . Values are means \pm S.E.M. of at least three independent experiments performed in triplicate. The ratio of the mean IC_{50} determined in the absence of Ca^{2+} over those determined in the presence of Ca^{2+} is indicated.

	Ca^{2+}	EGTA	EGTA/ Ca^{2+}
Agonists	IC_{50} (μM)	IC_{50} (μM)	$\text{IC}_{50}/\text{IC}_{50}$
GABA	238 ± 28	227 ± 55	0.95
APPA	86.0 ± 4.0	140 ± 3	1.63
Baclofen	28.8 ± 5.4	31.2 ± 0.9	1.08
Antagonists	IC_{50} (nM)	IC_{50} (nM)	$\text{IC}_{50}/\text{IC}_{50}$
CGP64213	4.48 ± 0.84	7.04 ± 2.11	1.57
CGP54626A	102 ± 26	123 ± 21	1.21
CGP56999A	1.43 ± 0.37	2.77 ± 0.94	1.93

effect on the GABA and APPA affinities on this mutant receptor. These data demonstrate that Ser269 in the GABA_BR1 subunit is responsible for the Ca²⁺ effect on the heteromeric GABA_B receptor. In agreement with an important role for Ser269 in GABA_BR1, this specific residue is conserved in both the *Caenorhabditis elegans* and *Drosophila melanogaster* GABA_BR1 receptors (Fig. 3). In contrast, Ser247 and Ser268, which we show are not important for the Ca²⁺-effect on GABA_BR1 are not conserved in the *C. elegans* and *D. melanogaster* sequences (Fig. 3).

Comparison with CaSR and mGluRs. Within the different members of the family 3 GPCRs, Ca²⁺ has been shown to regulate the activity of the CaSR and of some mGluRs. In the case of the CaSR, Ca²⁺ seems to be sufficient to activate the receptor (Brown et al., 1993; Hammerland et al., 1999). In the case of mGluR1, mGluR3, and mGluR5, Kubo et al. proposed that Ca²⁺ also directly activates these receptors (Kubo et al., 1998). However, other authors did not observe such a direct activation of mGluR1 by Ca²⁺; rather, they found that Ca²⁺ increases the potency of glutamate to activate the receptor (Saunders et al., 1998). These later observations are not in contrast to those reported by Kubo et al., because it is possible that in Kubo's experiments, the extracellular glutamate concentration was too low to activate the receptor in the absence of Ca²⁺, but became effective upon addition of Ca²⁺. Ca²⁺ seems, therefore, to exert a similar action on some mGluRs and GABA_B receptors acting as an allosteric regulator and increasing the potency of some agonists. However, the apparent affinity of Ca²⁺ on mGluRs and GABA_B receptors is quite different, being in the millimolar range for mGluRs (Kubo et al., 1998; Saunders et al., 1998) and around 37 μ M for GABA_B receptors, suggesting that Ca²⁺ may not interact with these two receptor types in the same way.

Possible Mode of Action of Ca²⁺ on GABA_BR1. Ca²⁺ has also been shown to act on the extracellular leucine/isoleucine/valine binding protein (LIVBP)-like domain of the CaSR (Hammerland et al., 1999). Recently, Ser170 has been reported to play a critical role for the action of Ca²⁺ (Bräuner-Osborne et al., 1999). Interestingly, this residue aligns with Ser269 of GABA_BR1a (Fig. 3). However, the difference in potencies of Ca²⁺ on these two receptors (37 μ M on GABA_BR and 3 mM on CaSR), plus the observation that Ca²⁺ is sufficient to activate CaSR but not GABA_BR, suggest that Ca²⁺ does not act similarly on these two receptors.

In mGluRs, the absence of effect of Ca²⁺ on mGluR2 allows the identification of a single residue conserved in mGluR1, mGluR3, and mGluR5, and responsible for the Ca²⁺-effect on these receptors (Kubo et al., 1998). This residue is Ser166 in mGluR1, which is replaced by an Asp in mGluR2 (see Fig. 3). Ser166 is very close to the glutamate binding site, because the α -carboxylic and α -amino groups of glutamate have been proposed to interact with Ser165 and Thr188, respectively (O'Hara et al., 1993) (Fig. 3). In GABA_BR1a, Ser246 aligns with Ser165 of mGluR1 and has been reported to play a critical role for the binding of GABA and other GABA_B ligands (Galvez et al., 1999). These two residues align with Ser79 of LIVBP (Fig. 3), which forms a hydrogen bond with the α -carboxylic group of leucine in the bacterial protein (Sack et al., 1989), suggesting that the glutamate and GABA binding sites in mGluRs and GABA_BR1a may be similar to that of the leucine binding site in LIVBP. Although Ser166 of

mGluR1 is conserved in both mammalian GABA_BR1a (Ser247) and GABA_BR2 (Ser137), this residue is not involved in the Ca²⁺ effect on the GABA_B receptor. Indeed, as summarized above, our data indicate that Ser269 of GABA_BR1a is responsible for the Ca²⁺ effect. Ser269 of GABA_BR1a aligns with Thr188 of mGluR1 and Thr102 of LIVBP (Fig. 3). In mGluR1, Thr188 has been shown to interact with glutamate (O'Hara et al., 1993), and in LIVBP, Thr102 forms a hydrogen bond with both the α -amino and α -carboxylic groups of leucine (Sack et al., 1989). Because GABA lacks the α -amino group common to both leucine and glutamate, two possible modes of action of Ca²⁺ on the GABA_B receptor can be proposed. Ca²⁺ could compensate the lack of the α -amino group of GABA, interacting with Ser269 and allowing a better positioning of the carboxylic group of GABA for its proper interaction with Ser246. Alternatively, Ca²⁺ may not interact directly with Ser269 but may affect the position of its OH group, allowing the formation of an additional hydrogen bond with the carboxylic group of GABA. Further experiments are required to clarify the mode of action of Ca²⁺ on the GABA_B receptor.

The observation that baclofen does not require Ca²⁺ for high-affinity binding on GABA_BR1 may be explained in two ways. One possibility is that, because of the additional chlorophenyl group of this molecule, baclofen does not interact the same way as GABA in GABA_BR1a. Alternatively, the presence of the chlorophenyl group may not allow the binding of Ca²⁺ on the receptor.

Possible Physiopathological Relevance of the Ca²⁺-Effect on GABA_B Receptors. The potency of Ca²⁺ on both CaSR and mGluRs is in the millimolar range (Brown and Hebert, 1997; Kubo et al., 1998); therefore, it is in the range of the Ca²⁺ concentrations found in blood and in cerebrospinal fluid. Accordingly, the Ca²⁺-sensing function of both CaSR and mGluRs may play important physiological roles. In the case of the CaSR, this has been pointed out by identifying many point mutations in the CaSR gene that affect the potency of Ca²⁺ to activate this receptor, and that are associated with genetic diseases affecting the regulation of calcemia (Pollak et al., 1993; Pollak et al., 1994; Brown and Hebert, 1997). In the case of the GABA_B receptor, we found that the EC₅₀ value for Ca²⁺ is 37 μ M, such that a significant shift of the GABA dose-response curve can only be detected when the extracellular Ca²⁺ concentration was lowered to 100 μ M. In the brain, such low extracellular Ca²⁺ concentrations can be reached only under particular conditions, such as activation of glutamate receptors, especially those of the NMDA type (Pumain et al., 1983; Pumain and Heinemann, 1985; Pumain et al., 1987), or after epileptic seizure (Pumain et al., 1983; Heinemann et al., 1986; Armand et al., 1995). If this occurs in the vicinity of the GABA_B receptors, it will result in a decreased activity of GABA_B receptors, thereby preventing this receptor from being activated by GABA. Because GABA_B receptors regulate both glutamatergic and GABA-ergic synaptic transmission, it is difficult to predict whether such an effect will facilitate or inhibit the development of the seizure.

In conclusion, we show here that Ca²⁺ ions are required for the high-affinity binding of some but not all GABA_B ligands, and we show that Ser269 of the GABA_BR1 subunit is involved in this Ca²⁺-effect. Because of the high potency of Ca²⁺ for the GABA_B receptor, this effect is unlikely to have

an important regulatory role. However, one should take into account the presence of Ca^{2+} in the $\text{GABA}_B\text{R1}$ binding site for the understanding of the activation process of GABA_B receptors.

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