Evidence for a Reduction of Coupling between GABA\textsubscript{A} Receptor Agonist and Ionophore Binding Sites by Inorganic Phosphate*

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(Accepted February 17, 2005)

\[^{35}S\]TBPS binding to the GABA\textsubscript{A} receptor ionophore binding site is anion dependent. Using autoradiography on rat brain sections, we show that permeabilities of anions through the receptor channel correlate with their efficiencies to promote basal \[^{35}S\]TBPS binding. Phosphate made an exception as it induced more binding than expected from its permeability. Well-permeable anions (chloride, nitrate, formate) allowed \[^{35}S\]TBPS binding to be effectively displaced by 1 mM GABA, whereas low-permeable anions (acetate, phosphate, propionate) markedly prevented this GABA effect, especially in the thalamus, the transition from the high to the low GABA effect being between formate and acetate. In the presence of phosphate, GABA enhanced \[^{3}H\]flunitrazepam binding to benzodiazepine site of recombinant \(\alpha1\beta2\gamma2\) receptors with the same efficacy but lower potency as compared to the presence of chloride, whereas \[^{35}S\]TBPS binding was abnormally modulated by GABA. These results suggest that inorganic phosphate affects coupling between agonist and ionophore sites in GABA\textsubscript{A} receptors.

KEY WORDS: Binding sites; brain regions; GABA\textsubscript{A} receptor; inorganic phosphate; recombinant receptors.

INTRODUCTION

\(\gamma\)-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain, and its fast actions are mediated by type A (GABA\textsubscript{A}) receptors. GABA\textsubscript{A} receptors belong to the family of ligand-gated ion channels, implying that the GABA binding site is located on the same complex as the anion permeable pore. In addition to the agonist binding site(s), GABA\textsubscript{A} receptors comprise binding sites for several important therapeutics, such as benzodiazepines, barbiturates and volatile anesthetics (1), prescribed to treat, for example, sleep, anxiety and epileptic disorders, and to induce and maintain general anesthesia. Pentameric receptor complexes are formed by assembly of subunits from a repertoire of at least 16 members, namely \(\alpha1–6, \beta1–3, \gamma1–3, \delta, \varepsilon, \theta, \pi\) (2). The multiplicity of native GABA\textsubscript{A} receptors is regulated in a well-known temporal and spatial subunit gene expression (3–5), but with poorly understood rules of assembly. Anyhow, recent studies have presented evidence for a preferred assembly of 2\(\alpha\) × 2\(\beta\) × 1\(\gamma\) subunits in most native receptor complexes (6,7), with \(\delta\), substituting \(\gamma\) (8,9) and perhaps \(\beta\), \(\varepsilon\), \(\theta\) and \(\pi\) in some receptors.

Whereas all the GABA\textsubscript{ergic} drugs in clinical use are positive modulators (1), there is also a

\* Special issue dedicated to Simo S. Oja.
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heterogenous group of compounds that bind to a distinct binding site in the receptor complex-associated ionophore, block GABA-induced chloride influx, and thus cause convulsions. Most of these compounds were developed as insecticides or anthelmintic, but two of these convulsants, picrotoxinin and t-butylbicyclophosphorothionate (TBPS), are frequently used experimentally. Picrotoxinin is typically employed in electrophysiological studies because of the fast onset of its block [ms range (10)], which is strikingly different from that of TBPS [about 30 min to peak inhibition (11)]. Regardless of the slow association, [35S]TBPS binding can be used to image the convulsant/ionophore sites (12,13). [35S]TBPS binding is absolutely anion dependent (12,13), and anion permeability though the GABA_A receptor ionophore bears a direct relationship to [35S]TBPS affinity (14). Since changes in the chloride concentration affect mainly rate constants of [35S]TBPS binding association, but not dissociation (15), Maksay (16) has speculated that anions are needed for the formation of the binding site for [35S]TBPS. In the presence of chloride and absence of GABA, [35S]TBPS binds to picrotoxinn-sensitive sites slowly, and equilibrium is reached in 3 h (17). [35S]TBPS dissociates in a slow monophasic way with a dissociation constant of 88 min (17).

High [35S]TBPS binding under basal conditions is considered to represent resting receptors, whereas dissociation by GABA and other positive modulators is thought to be a biochemical measure of receptor activation (18). Usually low micromolar GABA leads to a total displacement of [35S]TBPS binding. Anyhow, using recombinant receptors expressed in different expression systems, it is possible to produce such [35S]TBPS binding that is only partly, if at all, displaced by even high GABA concentrations [β3 (19); α6β2/3 (20,21); α2β1/3γ2/3, α3β3γ2/3, α5β3γ2 (22)]. We and others have shown that certain native GABA_A receptor populations have similar properties (17,20,23). We named the residual [35S]TBPS binding in the presence of 1 mM GABA “GABA-insensitive [35S]TBPS binding (GIS).” This atypical coupling between agonist and convulsant binding sites represents roughly 5% of the receptors, and has been mainly located in the thalamus and cerebellar granule cell layer of human, rat, mouse and chicken brain (23). Studies with genetically engineered mice suggest that extrasynaptic receptors devoid of γ2 subunit display GIS-[35S]TBPS binding in the brain (21,24).

In the standard [35S]TBPS autoradiographic procedure chloride is used as the main anion (17,25,26). There is a positive correlation between the anion permeability through the GABA_A receptor channel and the potency of an anion to enhance [35S]TBPS binding (i.e. affinity) (14). Anion permeability correlates with the anion size, i.e. small anions are more permeable than large. In the present study we probed the effects of anion size on coupling between GABA and [35S]TBPS binding sites. We hypothesized that well-permeable anions would allow efficient receptor conformational changes by GABA leading to dissociation of [35S]TBPS binding, since high GABA concentration and high anion permeability would lead to vivid channel activity. Vice versa, larger and less permeable anions would prevent coupling because of poor permeability and thus slower traffic.

**EXPERIMENTAL PROCEDURES**

**Recombinant GABA_A Receptor Expression for Electrophysiological Recordings**

Transfection with recombinant α1β3γ2 GABA_A receptors was carried out as described in detail (20, 22). HEK-293 cells were passaged and plated on 12-mm glass coverslips in 9.6-cm tissue culture dishes filled with 10 ml of Minimum Essential Medium (MEM, Gibco, Gaithersburg, MD) supplemented with 158 mg/l sodium bicarbonate, 2 mM glutamine (Gibco), 100 U/ml penicillin-streptomycin (Gibco), and 10% fetal calf serum (Gibco). These cultures were maintained at 37°C in a humidified 95% O2/5% CO2 atmosphere for 2–3 days. After incubation, cells were transfected using the phosphate precipitation method with rat α1, β3 and γ2 subunit cDNAs in eukaryotic expression vectors (27,28). For the identification of transfected cells 1 μg/plate of eGFP cDNA (Clontech, Palo Alto, CA) was added. Two days after transfection single coverslips containing HEK 293 cells were placed in a recording chamber mounted on the movable stage of an inverted fluorescence microscope (Olympus IX70, Olympus Optical Co. GmbH, Hamburg, Germany) and perfused with a defined standard saline solution containing (in mM): 130 NaCl, 5.3 KCl, 2 CaCl2, 2 MgSO4, 1.1 glucose, 5 sucrose, and 10 HEPES, pH adjusted to 7.4 with 30 mM NaOH and an osmolarity of about 310 mOsm. Solutions for anion replacement experiments had half of the NaCl (65 mM) substituted with sodium salts of nitrate (NO3−), bromide (Br−), formate (COO−), bicarbonate (HCO3−), acetate (CH3COO−) or propionate (CH3CH2COO−). Ligand-mediated membrane currents of cells identified by their GFP fluorescence were studied in the whole-cell configuration of the patch-clamp technique (29). Patch-clamp pipettes were pulled from hard borosilicate capillary glass (0.5 mm ID, 1.5 mm OD, Vitrex, Science Products GmbH, Hofheim, Germany) using a horizontal puller (model P-97, Sutter Instrument Co., Novato, CA) in a multi-stage process. The pipettes had an initial resistance of 3–5 MΩ when filled with a solution containing (in mM): 70 CsCl, 50 CsOH, 20 CsF, 2 CaCl2, 2 MgCl2, 10 EGTA, 3.1 K+ATP, 0.4 Na2GTP and 10 HEPES, pH 7.35. The junction potential between the pipette and the external solution was less than 2.3 mV and therefore ne-