Interaction of Non-competitive Blockers within the \(\gamma\)-Aminobutyric Acid Type A Chloride Channel Using Chemically Reactive Probes as Chemical Sensors for Cysteine Mutants\(^*\)

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Selected channel-lining cysteine mutants from the M2 segment of rat \(\alpha_1\) \(\gamma\)-aminobutyric acid (GABA\(_A\)) type A receptor subunit, at positions 257, 261, 264, and 272 were co-expressed with \(\beta_1\) and \(\gamma_2\) subunits in Xenopus oocytes. They generated functional receptors displaying conductance and response to both GABA and picrotoxinin similar to the wild type \(\alpha_1\beta_1\gamma_2\) receptor. Three chemically reactive affinity probes derived from non-competitive blockers were synthesized to react with the engineered cysteines: 1) dithiane bis-sulfone derivative modified by an isothiocyanate function (probe A); 2) fiprole derivatives modified by an \(\alpha\)-chloroketone (probe B) and \(\alpha\)-bromoketone (probe C) moiety. These probes blocked the GABA-induced currents on all receptors. This blockade could be fully reversed by a washing procedure on the wild type, the \(\alpha_1\)T261C/\(\beta_1\gamma_2\) and \(\alpha_1\)L284C/\(\beta_1\gamma_2\) mutant receptors. In contrast, an irreversible effect was observed for all three probes on both \(\alpha_1\)V257C/\(\beta_1\gamma_2\) and \(\alpha_1\)S272C/\(\beta_1\gamma_2\) mutant receptors. This effect was probe concentration-dependent and could be abolished by picrotoxin and/or \(\beta\)-butyl bicyclophosphorothionate. These data indicate a major interaction of non-competitive blockers at position 257 of the presumed M2 segment of rat \(\alpha_1\) subunit but also suggest an interaction at the more extracellular position 272.

\(\gamma\)-Aminobutyric acid type A (GABA\(_A\)) receptors exert their inhibitory effect in the central nervous system of vertebrates by regulating a chloride-sensitive channel which is very likely centered within a protein transmembrane heteropentameric subunits complex (1–5). The existence of \(\alpha_1\), \(\alpha_2\), \(\beta_1\), \(\beta_2\), \(\gamma_2\), and \(\delta\) subunits in addition to splicing variants, suggests a large diversity in the constitution of heteropentameric isoforms allowing a subtle tuning of the action of this neurotransmitter (6–8). However, it has been proposed that a restricted number of combinations condition the functioning of this receptor and it is assumed that the \(\alpha_1\)\(\beta_2\)\(\gamma_2\) represents the major adult isoform (9). GABA\(_A\) receptors serve as the target for several classes of molecules including important neuroactive drugs such as benzodiazepines, barbiturates, and neurosteroids. In contrast, only three receptor subunits have been cloned from insects up to now, RDL (10), \(\beta_1\) (11), and GRD (12) leading to an apparently less complex situation for their structural assembly. Of particular interest are the action of non-competitive antagonists which are presumed to interact within the GABA receptor chloride channel leading to powerful insecticidal properties when presenting a selectivity for insect GABA receptor (13–16).

To investigate, at a molecular level, the interaction of non-competitive GABA antagonists with the chloride channel associated to the GABA\(_A\) receptor, we defined an approach which uses chemically reactive non-competitive blockers (NCBs) as chemical sensors for cysteine mutants on the rat \(\alpha_1\) GABA receptor subunit. This strategy was derived from the extensive work of Akbas and co-workers on several ionic channels including the chloride channel associated to the GABA\(_A\) receptor (17) which identified the receptor channel-lining residues using a cysteine accessibility method (18). Selected channel-lining cysteine mutants from the \(\alpha_1\) rat subunit, at positions 257, 261, 264, and 272, respectively, when co-expressed with \(\beta_1\) and \(\gamma_2\) subunits in Xenopus oocytes, were probed for their ability to react covalently with several chemically modified NCBs derived from dithiane bis-sulfones (19, 20) and the insecticide fipronil (21). The reactive chemical functions were either aromatic isothiocyanates or \(\alpha\)-chloro- and \(\alpha\)-bromoketone fiproles. The formation of a selective covalent bond between the cysteine mutant and the reactive NCB, protectable by reference NCBs as PTX or TBPS, allows positioning in a spatial proximity of the cysteine residue with the reactive group of the NCB and most importantly, has made it possible to discriminate effects due to an allosteric interaction induced by the mutation.

In this study we present evidence that the synthesized reactive NCB probes had a fully reversible effect on the wild type receptor as well as on the \(\alpha_1\)T261C/\(\beta_2\gamma_2\) and \(\alpha_1\)L284C/\(\beta_2\gamma_2\) mutant receptors. In contrast, a selective irreversible effect could be demonstrated for the \(\alpha_1\)V257C/\(\beta_2\gamma_2\) and \(\alpha_1\)S272C/\(\beta_2\gamma_2\) mutant receptors. Although, the interaction of NCBs such as PTX at position 257 of the M2 helix was already postulated (22), a specific interaction at position 272 of the helix, close to the entrance of the channel, was not described and if it occurs, would allow new insight on the interaction of NCBs within this ionic channel. In addition, the formation of the covalent bonds, especially at position 257, suggests a positioning of the reactive ligands, in an oriented manner within the channel.

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\(\|$\) The abbreviations used are: GABA\(_A\), \(\gamma\)-aminobutyric acid type A; NCB, non-competitive blocker; PTX, picrotoxinin; CFFR, calcium-free frog Ringer’s solution; TBPS, tert-butyl-bicyclophosphorothionate; WT, wild type.
interaction of NCBs within the GABA<sub>A</sub> receptor channel

**Experimental Procedures**

**Synthesis of Affinity Probes**—The dithiane bis-sulfonyl isothiocyanate A was synthesized starting from the previously described aromatic difluorooazido derivative (20) by catalytic hydrogenation followed by treatment of the amino group by thiophosgene in acetone to the desired aromatic difluoroisothiocyanate with a global yield of 27% for these two steps. Probes B and C were a gift from Rhône-Poulenc Ag Co. (Research Triangle Park, North Carolina).

**GABA<sub>A</sub> Receptor Subunit cDNA Clones**—The cDNA encoding rat α1, α1V257C, α1T261C, α1L284C, α1S227C, β1, and γ2 in pBluescript SK(−) plasmids were obtained from Prof. M. H. Akabas (Columbia University, New York). Site-directed mutagenesis was performed as described (23) to construct the α1V257S cDNA using the following primers: 5′-CACCGAAGAATCTTCTTTGGACG-3′ and 5′-GCTCACTCTCAAAGGAGTCTCTGAG-3′ in the polymerase chain reaction with the α1 subunit cDNA inserted in a pBluescript SK(−) plasmid (Stratagene) as a template. The underlined position indicates the position of the Val to Ser mutation. After purification of the DNA fragments, the mutation was checked by DNA sequencing.

**Expression of GABA Receptor Subunits**—For in vitro mRNA transcription, the plasmids containing the α1 and β1 subunits were linearized with HindIII and the γ2 subunit was linearized with NotI. mRNA was transcribed in vitro by T<sub>7</sub>, T<sub>8</sub>, or T<sub>18</sub> (α1 an γ2) polymerase using the Ambion (Austin, TX) mMessage mMachine kit. Stage V and VI Xenopus laevis oocytes were harvested and defolliculated as described (24). One day after the oocytes were harvested, they were injected with 20 ng of total mRNA (mixed in the subunit α1β1γ2 ratio 1:1:1) dissolved in 50 nl of nuclease-free water. After injection, the oocytes were incubated 3–6 days at 18°C in OR-3 (1:2 dilution of L-15 Leibovitz media, 1 µM glutamine, gentamycin (100 µg/ml<sup>−1</sup>), 15 µM Heps adjusted to pH 7.6 with NaOH) before recordings.

**Electrophysiological Recordings**—GABA-induced currents were recorded from individual oocytes under two-electrode voltage clamp at a holding potential of −80 mV. Microelectrodes were filled with 3 M KCl and had a resistance of 0.5–3 megohm. The ground electrode was activated with NaOH before recordings.

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**RESULTS**

**Pharmacological Characterization of the Recombinant Receptors**—GABA-induced currents were recorded on wild type (α1β1γ2) and mutant recombinant receptors (α1V257Cβ1γ2, α1T261Cβ1γ2, α1L284Cβ1γ2, α1S272Cβ1γ2, and α1V257Sβ1γ2) (Fig. 1), expressed in oocytes. The selected cysteine mutants showed currents very similar to the WT, in accordance with the described results with even slightly less pronounced differences (17), while the serine mutant at position 257 showed a 2-fold increase in the GABA-induced currents (Table I).

The sensitivity to the GABA response and the effect of the reference NCB PTX were tested for the different recombinant receptors. The observed EC<sub>50</sub> and IC<sub>50</sub> values (Table I) further indicated that the binding properties for GABA and PTX were only slightly affected by the various cysteine mutations, while the α1V257S mutant showed an increased affinity for the GABA molecule (EC<sub>50</sub> 2 µM versus 17 µM for the WT), in agreement with the previously observed GABA-induced current increase.

**Biochemical and Pharmacological Evaluation of the Reactive Probes**—The affinities of the reactive probes A, B, and C (Fig. 1) for the NCB-binding site were estimated by a displacement of <sup>[3H]1-(4′-Ethynylphenyl)-4-propyl-2,6,7-trioxabicyclo[2,2,2]octane (EBOB) on rat brain and housefly membranes. The obtained IC<sub>50</sub> values (not shown), indicate affinities in the submicromolar range for the vertebrate receptor. A marked difference was noticeable on the rat brain membranes for the 4-acyl-pyrazole side chain substituent in the fiprole series, i.e. IC<sub>50</sub> values for B and C were 0.2 and 1 µM, respectively. The effect of the three probes on recombinant α1β1γ2 WT receptors expressed in Xenopus oocytes was analyzed by binding the GABA-induced chloride currents after applying excess of the probes (100 µM probe A and 10 µM fiprole derivatives B and C) (Table II). Eighty to 90% inhibition of the GABA-induced currents were obtained for these probes and this effect could be fully reversed by successive washings.

The following sequence of perfusion adapted from a previously described procedure (17) was used to determine an irreversible effect: 100 µM GABA 10 s, CFFR 3 min; 100 µM GABA 10 s, CFFR 3 min; 100 µM A, 10 µM B, or 10 µM C, 1 min; CFFR 3 min; 100 µM GABA 10 s, CFFR 3 min; 100 µM GABA 10 s, CFFR 6 min; 100 µM GABA 10 s, CFFR 5 min; 100 µM GABA 10 s.

To test the ability of NCBs to protect the engineered cysteines from modification by the probes, we used the following sequence of perfusion solutions: 100 µM GABA 10 s, CFFR 3 min, 100 µM GABA 10 s, CFFR 3 min, 100 µM PTX or 10 µM TBPS 1 min, 100 µM A, 10 µM B, or 10 µM C + 100 µM PTX or 10 µM TBPS 1 min, CFFR 3 min, 100 µM GABA 10 s, CFFR 3 min, 100 µM GABA 10 s, CFFR 3 min, 100 µM GABA 10 s.

The average of the two peak currents before probe application was used as the control response. The fractional effect at the different times after application was taken as (1 − [I<sub>GABA, before</sub>−I<sub>GABA, after</sub>]/[I<sub>GABA, before</sub>]). The irreversible effect was calculated by comparing the average of the two peak currents 3 and 9 min after probe application. Similar results were obtained on 3–6 single oocytes for each probe and each receptor.


**Interaction of NCBs within the GABA\(_\alpha\) Receptor Channel**

**Table I**

Pharmacological evaluation of the expressed recombinant receptors

| Recombinant receptor | Current | GABA EC\(_{50}\) | PTX IC\(_{50}\) |
|----------------------|---------|-----------------|----------------|
|                      | nA      | \(\mu\)m        | \(\mu\)m        |
| WT                   | 873 \(\pm\) 17 | 17 \(\pm\) 3 | 1.0 \(\pm\) 0.3 |
| \(\alpha_V257C\beta_1\gamma_2\) | 892 \(\pm\) 88 | 11 \(\pm\) 2 | 1.6 \(\pm\) 0.5 |
| \(\alpha_T261C\beta_1\gamma_2\) | 640 \(\pm\) 36 | 33 \(\pm\) 9 | 1.4 \(\pm\) 0.3 |
| \(\alpha_L264C\beta_1\gamma_2\) | 726 \(\pm\) 125 | 18 \(\pm\) 5 | 3.0 \(\pm\) 1.8 |
| \(\alpha_S272C\beta_1\gamma_2\) | 987 \(\pm\) 56 | 24 \(\pm\) 8 | 1.0 \(\pm\) 0.4 |
| \(\alpha_V257S\beta_1\gamma_2\) | 2021 \(\pm\) 290 | 2 \(\pm\) 1 | 1.7 \(\pm\) 0.3 |

**Table II**

Reversible and irreversible inhibition of GABA induced currents by the affinity probes on WT and mutant GABA\(_\alpha\) receptors

The percent inhibition, represented % reversible (% irreversible), induced by probes A, B, or C were determined from X. laevis injected oocytes according to the procedures described under "Experimental Procedures." Data indicate the mean \(\pm\) S.E. of three to five individual oocytes.

| Recombinant receptor | A, 100 \(\mu\)m | B, 10 \(\mu\)m | C, 10 \(\mu\)m |
|----------------------|-----------------|----------------|----------------|
| WT                   | 81 \(\pm\) 1 (0) | 90 \(\pm\) 1 (0) | 82 \(\pm\) 2 (0) |
| \(\alpha_V257C\beta_1\gamma_2\) | 80 \(\pm\) 2 (69 \(\pm\) 2) | 88 \(\pm\) 3 (50 \(\pm\) 2) | 82 \(\pm\) 2 (59 \(\pm\) 2) |
| \(\alpha_T261C\beta_1\gamma_2\) | 83 \(\pm\) 2 (0) | 85 \(\pm\) 2 (0) | 83 \(\pm\) 1 (0) |
| \(\alpha_L264C\beta_1\gamma_2\) | 81 \(\pm\) 2 (10) | 87 \(\pm\) 2 (0) | 85 \(\pm\) 3 (0) |
| \(\alpha_S272C\beta_1\gamma_2\) | 84 \(\pm\) 3 (40 \(\pm\) 5) | 88 \(\pm\) 2 (20 \(\pm\) 1) | 85 \(\pm\) 1 (40 \(\pm\) 2) |
| \(\alpha_V257S\beta_1\gamma_2\) | ND\(^a\) | ND | 79 \(\pm\) 3 (0) |

\(^a\) ND; not determined.

**DISCUSSION**

The GABA\(_\alpha\) receptors have been proposed to be a protein complex composed of (hetero)pentameric transmembrane subunits assembled comprising a central cavity defining an ionic channel (3, 6, 25). A series of neurotoxic insecticides, including the toxin PTX, or synthetic compounds such as polychlorocycloalkanes and fipronil, inhibit GABA-induced currents by binding to the NCB site(s) of the GABA\(_\alpha\) receptor complex of vertebrates and invertebrates with variable efficacy (14, 15, 26). By analogy to the nicotinic acetylcholine receptor, the M2 membrane-spanning segment of the different GABA receptor subunits have been suggested to line the channel pore (27), and numerous site-directed mutagenesis experiments achieved within this segment of vertebrate or invertebrate receptors have demonstrated an effect on the binding of the NCBS. A typical example being the natural point mutation A302S in the Drosophila melanogaster GABA receptor (RDL subunit) conferring high levels of resistance to PTX and dieldrin (28), this residue being homologous to the rat \(\alpha_{1} Val^{297}\). Using the cysteine accessibility approach, Akabas and co-workers (22) stud-
versed versus the presumed M2 helix of the human GABA receptor. The mutation of the conserved leucine residue at the center of the presumed M2 membrane spanning segment were found to be the only amino acids from the presumed membrane spanning segment M2 of the different GABA receptor subunits, the cytoplasmic position at the level of Val257 of rat α1 subunit, corresponding to the natural mutation in Drosophila Rd1 A302S subunit and the Pro309 in human ρ1 subunit, represents a crucial position in the NCB interaction with various GABA receptors.

The aim of our approach, which combines a site-directed labeling method to noninvasive site-directed mutagenesis experiments, is to induce a specific irreversible reaction between a reactive electrophilic affinity ligand analog and a nucleophilic cysteine mutant. The affinity probes which were synthesized derived from two structurally unrelated NCBS having insecticidal properties, the dithiane bis-sulfone and the fipronil series (13, 15, 19) which were chemically modified by an isothiocyanate function or α-chloro and α-bromo moiety leading to probes A, B, and C, respectively. When tested independently for their chemical reactivity toward nucleophilic amino acids, the cysteine residues were found to be the only amino acids reacting efficiently and instantaneously with our probes at neutral pH (not shown). Therefore we generated cysteine mutants within the α subunit of the GABA receptor to ensure the chemical reactivity. We selected GABA receptor channel-lining residues, respectively, at positions 257, 261, 264, and 272, which were identified as controlling the sensitivity to PTX inhibition after expression in oocytes (36). Taken together, these results support a mechanism by which PTX blocks the ionic GABA channel, presumably through binding in the channel lumen without, however, rejecting conclusively allosteric mechanism alternatives. Among the amino acids from the presumed membrane spanning segment M2 of the different GABA receptor subunits, the cytoplasmic position at the level of Val257 of rat α1 subunit, corresponding to the natural mutation in Drosophila Rd1 A302S subunit and the Pro309 in human ρ1 subunit, represents a crucial position in the NCB interaction with various GABA receptors.

The action of PTX on the protection of the chemical modification by cysteine-specific permeants (cationic and anionic) at positions 257 and 261 of the rat α1 subunit in α1β1γ2 recombinant receptors. They showed a protective effect induced by PTX only at the more cytoplasmic position 257 and this effect was complete, only in an open GABA channel configuration. This action was proposed to result from a direct interaction of PTX in the GABA channel at the level of residue 257, this interaction becoming more effective in an open channel receptor conformation, rather than an allosteric effect induced by PTX binding outside the channel. A series of mutants near the center of the presumed M2 helix residues were found to be the only amino acids reacting efficiently and instantaneously with our probes at neutral pH (not shown). Therefore we generated cysteine mutants within the α subunit of the GABA receptor to ensure the chemical reactivity. We selected GABA receptor channel-lining residues, respectively, at positions 257, 261, 264, and 272, which were identified as controlling the sensitivity to PTX inhibition after expression in oocytes (36). Taken together, these results support a mechanism by which PTX blocks the ionic GABA channel, presumably through binding in the channel lumen without, however, rejecting conclusively allosteric mechanism alternatives. Among the amino acids from the presumed membrane spanning segment M2 of the different GABA receptor subunits, the cytoplasmic position at the level of Val257 of rat α1 subunit, corresponding to the natural mutation in Drosophila Rd1 A302S subunit and the Pro309 in human ρ1 subunit, represents a crucial position in the NCB interaction with various GABA receptors.

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three probes induced a higher inhibition at position 257 over position 272 (Table II). The specificity of the inactivation was demonstrated by the protective effect against the inactivation exerted either by PTX (Fig. 2C) and/or TBPS (not shown). Finally, to assess that the covalent reaction occurred between the mutated cysteine and the reactive moiety of the probe, we performed two additional controls. We checked that no irreversible reaction occurred, either on the serine mutant αV257Sβ1γ2 after treatment with probes A, B, or C (Table II) or, between a fiprole derivative having a nonreactive 4-acetyl pyrazole side chain with the cysteine mutant αV257Cβ1γ2 (not shown). Clearly, the observed irreversible reactions required the vicinity of both cysteine and reactive probe and even more precisely, it required a spatial proximity between the cysteine side chain and the reactive moiety of the probe, allowing a tentative positioning of the probe within the channel cavity. While reaction at position 257 was fully expected, reinforcing previously described results and observations (23, 29), reaction at position 272, presumably very close to the extracellular side, in conjunction with the absence of reaction at the intermediate positions 261 and 264, represents a new result. The simplest tentative explanation to account for these results could be that the NCBs are “filtered” by a narrow part at the channel entrance (position 272), than passing by a more widely open intermediate part before reaching the site located deeply in the channel (position 257) and acting like a plug at this position. Such an overall structure could also explain why PTX does not protect the cysteine mutant at position 261 from the alkylation by the ionic permeants which are smaller molecules and used in large excess (22). Due to the strong hydrophobic character of the probes in conjunction with their respective potencies and the used concentrations, it seems very unlikely that the covalent reaction occurring at position 257 could result through an action of the probe coming from the inside of the cell, after crossing the membrane. For instance, such an explanation would be in contradiction with the fact that all three probes had a much stronger irreversible effect at position 257 over 272, also the efficiency of probe C (59% irreversible inhibition at position 257 at 10 μM) would be fully unexpected, knowing its high hydrophobic character and its moderate affinity for the NCB site (IC50 [3H]EBOB ~ 1 μM on rat membranes).

The proposed mode of action of the NCBs within the GABA receptor channel raises, however, different questions. The description of an ionic channel centered in the cleft of five straight helical transmembrane segments cannot explain satisfactorily our labeling results. They do not coincide with a structural description given for the nicotinic acetylcholine receptor, where the narrow part of the channel is proposed to be located at the middle of the transmembrane segments (37). The irreversible reaction observed at position 272 could either reflect a narrowing of the channel entrance, implying the contribution of other subunits at homologous positions, or it could suggest the existence of a recognition site located mainly on the α-subunit(s) and involving additional α subunit residues, e.g. residues from the putative membrane-spanning helix M1, as was proposed for the interaction of a NCB with the open state of the nicotinic acetylcholine receptor (38). Clearly, additional experiments are necessary to describe more precisely the NCB site on the GABA receptor but again these experiments can be undertaken in the light of our new approach combining cysteine mutants with our reactive probes.

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