Neurosteroids Allosterically Modulate Binding of the Anesthetic Etomidate to γ-Aminobutyric Acid Type A Receptors

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Photoaffinity labeling of γ-aminobutyric acid type A (GABAₐ) receptors (GABAₐR) with an etomidate analog and mutational analyses of direct activation of GABAₐR by neurosteroids have each led to the proposal that these structurally distinct general anesthetics bind to sites in GABAₐRs in the transmembrane domain at the interface between the β and α subunits. We tested whether the two ligand binding sites might overlap by examining whether neuroactive steroids inhibited etomidate analog photoaffinity labeling. We previously identified (Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) J. Neurosci. 26, 11599–11605) azietomidate photolabeling of GABAₐR α1Met-236 and βMet-286 (in αM1 and βM3). Positioning these two photolabeled amino acids in a single type of binding site at the interface of β and α subunits (two copies per pentamer) is consistent with a GABAₐR homology model based upon the structure of the nicotinic acetylcholine receptor and with recent αM1 to βM3 cross-linking data. Biologically active neurosteroids enhance rather than inhibit azietomidate photolabeling, as assayed at the level of GABAₐR subunits on analytical SDS-PAGE, and protein microsequencing establishes that the GABAₐR-modulating neurosteroids do not inhibit photolabeling of GABAₐR α1Met-236 or βMet-286 but enhance labeling of α1Met-236. Thus modulatory steroids do not bind at the same site as etomidate, and neither of the amino acids identified as neurosteroid activation determinants (Hosie, A. M., Wilkins, M. E., da Silva, H. M., and Smart, T. G. (2006) Nature 444, 486–489) are located at the subunit interface defined by our etomidate site model.

GABAₐ³ receptors (GABAₐR) are major mediators of brain inhibitory neurotransmission and participate in most circuits and behavioral pathways relevant to normal and pathological function (1). GABAₐR are subject to modulation by endogenous neurosteroids, as well as myriad clinically important central nervous system drugs including general anesthetics, benzodiazepines, and possibly ethanol (1–2). The mechanism of GABAₐR modulation by these different classes of drugs is of major interest, including identification of the receptor amino acid residues involved in binding and action of the drugs.

In the absence of high resolution crystal structures of drug-receptor complexes, the locations of anesthetic binding sites in GABAₐRs have been predicted based upon analyses of functional properties of point mutant receptors, which identified residues in the α and β subunit M1–M4 transmembrane helices important for modulation by volatile anesthetics (primarily α subunit) and by intravenous agents, including etomidate and propofol (β subunit) (3–5). Position βM2–15, numbered relative to the N terminus of the helix, functions as a major determinant of etomidate and propofol potency as GABA modulators in vitro and in vivo (6–8). By contrast, this residue is not implicated for modulation by the neurosteroids, potent endogenous modulators of GABAₐR (9).

Photoaffinity labeling, which allows the identification of residues in proximity to drug binding sites (10, 11), has been used to identify two GABAₐR amino acids covalently modified by the etomidate analog [³H]azietomidate (12): α1Met-236 within αM1 and βMet-286 within βM3. Photoaffilation of these residues was inhibited equally by nonradioactive etomidate and enhanced proportionately by GABA present in the assay, consistent with the presence of these two residues in a common drug binding pocket that would be located at the interface between the β and α subunits in the transmembrane domain (12). Mutational analyses identify these positions as etomidate and propofol sensitivity determinants (13–15).

A recent mutagenesis study (16) identified two other residues in GABAₐR αM1 and βM3 as critical for direct activation by neurosteroids, αThr-236 (rat numbering, corresponding to α1Thr-237, bovine numbering used here and by Li et al. (12)) and β1Yr-284. These residues were also proposed to contribute to a neurosteroid binding pocket in the transmembrane domain at the interface between β and α subunits, based upon their location in an alternative GABAₐR structural model that positioned these amino acids, and not α1Met-236 or βMet-286, at the subunit interface. For GABAₐRs and other members

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References

1. Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) J. Neurosci. 26, 11599–11605.

2. Hosie, A. M., Wilkins, M. E., da Silva, H. M., and Smart, T. G. (2006) Nature 444, 486–489.

3. The abbreviations and trivial names used are: GABA, γ-aminobutyric acid; GABAₐR, GABA type A receptor; azietomidate, 2-(3-methyl-3-yl)ethyl 1-(phenylethyl)-1H-imidazole-5-carboxylate; nACHR, nicotinic acetylcholine receptor; THDOC, tetrahydro-deoxy-corticosterone; EndoLys-C, endoproteinase Lys-C; HPLC, high pressure liquid chromatography; OPA, o-phthalaldehyde.

4. The GABAₐR α subunit protein sequence numbering is slightly different in those reports (12, 16) because mouse (16) mature sequence lacks one amino acid in the N-terminal found in cow sequence (12) (otherwise, they are exactly the same). The α1M1-Thr-236 in Hosie et al. (16) (refer to their Fig. 1a) is α1M1-Thr-237 here in Fig. 3 and in Li et al. (12) (refer to their Fig. 4c).
of the Cys-loop superfamily of neurotransmitter-gated ion channels, the transmembrane domain of each subunit is made up of a loose bundle of four $\alpha$ helices (M1–M4), with M2 from each subunit contributing to the lumen of the ion channel and M4 positioned peripherally in greatest contact with lipid, as seen in the structures of the *Torpedo* nicotinic acetylcholine receptor (nAChr) (17) and in distantly related prokaryote homologs (18). However, uncertainties in the alignment of GABA$_A$R subunit sequences relative to those of the nAChr result in alternative GABA$_A$R homology models (12, 19, 20) that differ in the location of amino acids in the M3 and M4 membrane-spanning helices and in the M1 helix in some models (16, 21).

If etomidate and neurosteroids both bind at the same $\beta/\alpha$ interface in the GABA$_A$R transmembrane domain, the limited space available for ligand binding suggests that their binding pockets may overlap and that ligand binding would be mutually exclusive. To address this question, we photolabeled purified bovine brain GABA$_A$R with $[^3]$H]azetomidate in the presence of different neuroactive steroids and determined by protein microsequencing whether active neurosteroids inhibited labeling of $\alpha$1Met-236 and $\beta$Met-286, as expected for mutually exclusive binding, or resulted in $[^3]$H]azetomidate photolabeling of other amino acids, a possible consequence of allosteric interactions. Active steroids failed to inhibit labeling and enhanced labeling of $\alpha$1Met-236, clearly indicating that the neurosteroid and the etomidate sites are distinct. Our GABA$_A$R homology model that positions $\alpha$1Met-236 and $\beta$Met-286 at the $\beta/\alpha$ interface, but not that of Hosie et al. (16), is also consistent with cysteine substitution cross-linking studies (20, 22), which define the proximity relations between amino acids in the $\alpha$M1, $\alpha$M2, $\alpha$M3, and $\beta$M3 helices, and these results support the interpretation that the two residues photolabeled by $[^3]$H]azetomidate are part of a single subunit interface binding pocket, whereas the steroid sensitivity determinants identified by mutagenesis neither are at the $\beta/\alpha$ subunit interface nor are contributors to a common binding pocket.

### EXPERIMENTAL PROCEDURES

**Solubilization and Purification of Bovine Brain GABA$_A$Rs**—GABA$_A$R was purified as reported previously (12) on a benzodiazepine Ro7/1986-1 affinity column. Detailed conditions for solubilization and purification are described there.

**Photoaffinity Labeling of Purified GABA$_A$R**—The peak $[^3]$Hmuscimol binding fraction (5 ml) from each affinity column elution was used for photolabeling without further dialysis or concentration. KCl was added to a final concentration of 0.1 m. An aliquot of GABA$_A$R (~40 nm $[^3]$Hmuscimol binding sites, 2.5 ml for preparative labeling or 0.14 ml for analytical) was equilibrated with $[^3]$Hazetomidate (final concentration, 2 $\mu$M) ± additional drugs, incubated for 1 h in the dark (4 °C), and irradiated (30 min, 365 nm). After photolabeling, the total protein was precipitated with methanol/chloroform, solubilized in sample loading buffer, and fractionated by SDS-PAGE. The resulting gel lanes were cut into 3-mm slices, and $[^3]$H incorporation was determined either directly by liquid scintillation counting (analytical labeling) or after elution from each slice into 1 ml of elution buffer (12). Aliquots of the eluted bands were assayed for $[^3]$H and pooled for proteolytic digestion.

Enzymatic Digestion, Reversed-phase HPLC, and Protein Microsequencing—Digestion with EndoLys-C (Princeton Separations) was carried out using 2.5 $\mu$g of protease for aliquots of GABA$_A$R subunit in 50 $\mu$l of digestion buffer for 3 days at 25 °C. Reversed-phase HPLC and peptide microsequencing were performed as described (12). $[^3]$H release in each cycle of Edman degradation was determined by liquid scintillation counting of fractions for 6 × 10 min. Standard error bars are included in the plots (see Figs. 1 and 2). To determine the amount of peptide sequenced, the individual residues were fit to the equation $I_0/0 = I_{s} (I_{s} + R_{a})$, where $I_{s}$ is the pmol detected in cycle $x$, $I_{0}$ is the starting amount of the peptide, and $R_{a}$ is the average repetitive yield. The range of specific residue photolabeling ((2×(pmu − $c_{pmu-}$))/($I_0/0 × R_{a}$)) was calculated using the $I_0/0 × R_{a}$ values calculated with 95% confidence. Treatment with o-phthalaldehyde (OPA) during sequencing was as described (12, 23). OPA reacts preferentially with primary rather than secondary amines (i.e. proline) and can be used at any cycle of Edman degradation to block sequencing of peptides not containing an N-terminal proline.

### RESULTS AND DISCUSSION

**Neuroactive Steroids Potentiate GABA$_A$R Photolabeling**—When purified bovine brain GABA$_A$Rs photolabeled with $[^3]$H]azetomidate were fractionated by SDS-PAGE, $[^3]$H incorporation was detected in GABA$_A$R subunit polypeptides of ~50–55 kDa that was inhibitable by >90% in the presence of nonradioactive etomidate and was shown to result from labeling of $\alpha$1Met-236 (or the homologous methionine in $\alpha$2, -3, -5) and $\beta$Met-286 (12). To test for interactions between azetomidate and neurosteroids, we photolabeled GABA$_A$R with $[^3]$H]azetomidate in the presence of 1 mM GABA (which enhances binding) and included (individually) in the labeling mixture three different neuroactive steroids: 5a-pregnan-3a-ol-20-one (allopregnanolone), tetrahydro-deoxy corticosterone (THDOC), or alphaxalone, at various concentrations (0, 0.1, 0.3, 1, 10 $\mu$M) in parallel. As shown in Fig. 1, all three steroids enhanced GABA$_A$R subunit labeling in a concentration-dependent manner. The concentration dependence of enhancement could be fit to logistic curves, yielding half-effect concentrations of 0.6, 0.9, and 0.8 $\mu$M for allopregnanolone, THDOC, and alphaxalone, respectively, under the experimental conditions employed. An inactive isomer of allopregnanolone, isopregnanolone (5a-pregnan-3b-ol-20-one), produced no modulation of labeling (Fig. 1A), demonstrating the pharmacological specificity of the observed enhancement of labeling.

**Neurosteroid Modulation of Photolabeling of $\alpha$1Met-236/ $\beta$Met-286**—Protein microsequencing established that the GABA$_A$R subunit photolabeling seen in the absence or presence of GABA resulted from photolabeling of $\alpha$1Met-236 and $\beta$Met-286 (12). As neurosteroids actually increased $[^3]$Hazetomidate photolabeling of GABA$_A$R subunits, we wanted to determine whether the presence of neurosteroids increased the

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5 As discussed in Li et al. (12), because of the high degree of sequence conservation for $\alpha$ subunit subtypes in the M1 region and $\beta$ subunit subtypes in the M3 region, including protease cleavage sites, the labeled amino acids cannot be assigned to specific subunit subtypes, and therefore they are referred to as $\alpha$1Met-236 (using the $\alpha$1 subunit numbering) and $\beta$Met-286.
labeling of either (or both) of those residues or potentially reduced labeling at those positions while enhancing labeling of other residues in α1M or βM3. Purified GABA\(_{\text{A}}\)R was photolabeled with \(\text{[}^3\text{H}\text{]}\)azetomidate on a preparative scale (110 pmol [\(\text{[}^3\text{H}\text{]}\)muscimol binding sites]) with \(\text{[}^3\text{H}\text{]}\)azetomidate in the presence of 1 mM GABA and concentrations of neurosteroid (for active steroids, 0, □; 100 nM, △; 300 nM, ○; 1 μM, ◊; 10 μM, ●) and for isopregnanolone, 10 μM, ●), and covalent \(\text{[}^3\text{H}\text{]}\)incorporation was determined by SDS-PAGE. Right panels, the concentration dependence of neurosteroid modulation, determined by normalizing the \(\text{[}^3\text{H}\text{]}\)in the GABA\(_{\text{A}}\)R subunit gel band(s) in the presence of neurosteroid (active steroids, ■; inactive steroid (isopregnanolone, ●) to that in the absence. The data points are the average range from two independent photolabeling experiments. The solid lines are fits of the data to a logistic function. Error bars indicate S.E.

**FIGURE 1. Neuroactive steroids (A, allopregnanolone; B, THDOC; C, alphaxalone) enhance \(\text{[}^3\text{H}\text{]}\)azetomidate photolabeling of GABA\(_{\text{A}}\)R, whereas isopregnanolone, an inactive allopregnanolone isomer, does not.** Left panels, GABA\(_{\text{A}}\)R (6 pmol) was photolabeled with \(\text{[}^3\text{H}\text{]}\)azetomidate in the presence of 1 mM GABA and concentrations of neurosteroid (for active steroids, 0, □; 100 nM, △; 300 nM, ○; 1 μM, ◊; 10 μM, ●) and for isopregnanolone, 10 μM, ●), and covalent \(\text{[}^3\text{H}\text{]}\)incorporation was determined by SDS-PAGE. Right panels, the concentration dependence of neurosteroid modulation, determined by normalizing the \(\text{[}^3\text{H}\text{]}\)in the GABA\(_{\text{A}}\)R subunit gel band(s) in the presence of neurosteroid (active steroids, ■; inactive steroid (isopregnanolone, ●) to that in the absence. The data points are the average range from two independent photolabeling experiments. The solid lines are fits of the data to a logistic function. Error bars indicate S.E.
Photoaffinity labeling with [3H]azietomidate led to the identification of α1Met-236 and βMet-286 in an unbiased search for photolabeled amino acids in GABA<sub>A</sub> R subunit digests (12). Although photoaffinity labeling is a powerful tool to identify amino acids contributing directly to drug binding sites in proteins, results can be biased because the photoreactive intermediate may react preferentially with only certain amino acid side chains, and there is also the possibility that highly reactive side chains distant from the binding site may be labeled (10, 11).

The aliphatic diazirine group in [3H]azietomidate results in a reactive aliphatic carbocation intermediate following irradiation, which reacts most efficiently with Asp, Glu, and Tyr and also with Gln, Ser, Cys, and His (23). The labeling of two relatively less reactive methionines with the etomidate analog (12) indicates that they might be the most reactive side chains within the binding site, but they are unlikely to be labeled as distant, highly reactive side chains. In addition, aliphatic carbocation intermediates are known to be highly reactive with water (t<sub>1/2</sub> ≈ 1 ns (25)), further limiting the possibility of diffusional encounters with reactive side chains distant from the binding site. That the photolabeling of α1Met-236 and βMet-286 was potentiated in the presence of GABA, fully inhibitable by etomidate (12), and inhibitable by propofol<sup>6</sup> provides further evidence that [3H]azietomidate is acting as a specific affinity label and that the photolabeling results provide a direct identification of two amino acids contributing to the etomidate binding site.

In contrast, Li et al. (12) were not able to determine whether or not the M2 residue (βAsn-265) identified as a determinant of etomidate and propofol sensitivity (6–8, 26) is labeled by [3H]azietomidate, although propofol does not protect βN265C from reaction with sulphydryl reagents, whereas it protects βM286C (14). In our homology model, this position (Fig. 3A) may be accessible both from the interior of the β subunit helix bundle and from the etomidate binding pocket at the β/α interface, and further studies are needed to determine whether it contributes directly to the etomidate binding site and/or is crucial for the coupling between anesthetic binding and GABA<sub>A</sub> R gating.

Our results and complementary cross-linking studies (22) indicate that the neurosteroid activation determinants in αM1 and βM3 are not contributing to a common binding pocket at the interface between β and α subunits, although Akk et al. (27) support the data of Hosie et al. (16), implicating residues in αM1 for steroid effects. Further studies with photoreactive neurosteroids (28) may be necessary to identify neurosteroid binding sites.

<sup>6</sup>G. D. Li, D. C. Chiara, J. B. Cohen, and R. W. Olsen, unpublished data.
REFERENCES

1. Olsen, R. W., and Sieghart, W. (2008) *Pharmacol. Rev.* **60**, 243–260
2. Hanchar, H. J., Dodson, P. D., Olsen, B. W., Otis, T. S., and Wallner, M. (2005) *Nat. Neurosci.* **8**, 339–345
3. Mihic, S. J., Ye, Q., Wick, M. J., Kolchicine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., Harris, R. A., and Harrison, N. L. (1997) *Nature* **389**, 385–389
4. Hemmings, H. C., Akabas, M. H., Goldstein, P. A., Trudell, J. R., Orser, B. A., and Harrison, N. L. (2005) *Trends Pharmacol. Sci.* **26**, 503–510
5. Franks, N. P. (2008) *Nat. Rev. Neurosci.* **9**, 370–386
6. Belelli, D., Lambert, J. J., Peters, J. A., Wafford, K., and Whiting, P. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11031–11036
7. Jurd, R., Arras, M., Lambert, S., Drexler, B., Siegwart, R., Crestani, F., Zaugg, M., Vogot, K. E., Ledermann, B., Antkowiak, B., and Rudolph, U. (2003) *FASEB J.* **17**, 250–252
8. Reynolds, D. S., Rosahl, T. W., Cirone, J., O’Meara, G. F., Haythornthwaite, A., Newman, R. J., Myers, J., Sur, C., Howell, O., Rutter, A. R., Attack, J., Macaulay, A. J., Hadingham, K. L., Hutson, P. H., Belelli, D., Lambert, J. J., Dawson, G. R., McKernan, R., Whiting, P. J., and Wafford, K. A. (2003) *J. Neurosci.* **23**, 8608–8617
9. Belelli, D., and Lambert, J. J. (2005) *Nat. Rev. Neurosci.* **6**, 565–575
10. Kotzyba-Hibert, F., Kapfer, I., and Goedlmer, M. (1995) *Angew. Chem. Int. Ed. Engl.* **34**, 1296–1312
11. Vodovozova, E. L. (2007) *Biochemistry (Mosk)* **72**, 1–20
12. Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) *J. Neurosci.* **26**, 11599–11605
13. Krasowski, M. D., Kolchicine, V. V., Rick, C. E., Ye, Q., Finn, S. E., and Harrison, N. L. (1998) *Mol. Pharmacol.* **53**, 530–538
14. Bali, M., and Akabas, M. H. (2004) *Mol. Pharmacol.* **65**, 68–76
15. Stewart, D., Desai, R., Cheng, Q., Liu, A., and Forman, S. A. (2008) *Mol. Pharmacol.* **74**, 1687–1695
16. Hosie, A. M., Wilkins, M. E., da Silva, H. M., and Smart, T. G. (2006) *Nature* **444**, 486–489
17. Unwin, N. (2005) *J. Mol. Biol.* **346**, 967–989
18. Hill, R. J., and Dutzler, R. (2008) *Nature* **452**, 375–379
19. Ernst, M., Bruckner, S., Boresch, S., and Sieghart, W. (2005) *Mol. Pharmacol.* **68**, 1291–1300
20. Jansen, M., and Akabas, M. H. (2006) *J. Neurosci.* **26**, 4492–4499
21. Yamakura, T., Bertaccini, E., Trudell, J. R., and Harris, R. A. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41**, 23–51
22. Bali, M., Jansen, M., and Akabas, M. H. (2009) *J. Neurosci.* **29**, 3083–3092
23. Ziebell, M. R., Nirthanan, S., Husain, S. S., Miller, K. W., and Cohen, J. B. (2004) *J. Biol. Chem.* **279**, 17640–17649
24. Ruesch, D., Zhong, H. J., and Forman, S. A. (2004) *J. Biol. Chem.* **277**, 20982–20992
25. McClelland, R. A. (2004) in *Reactive Intermediate Chemistry* (Moss, R. A., Platz, M. S., and Jones, M., Jr., eds), pp. 3–40, Wiley-Interscience, Hoboken, NJ
26. Siegwart, R., Jurd, R., and Rudolph, U. (2002) *FASEB J.* **17**, 250–252
27. Akk, G., Covey, D. F., Evers, A. S., Steinbach, J. H., and Mennerick, S. (2007) *Pharmacol. Ther.* **116**, 35–57
28. Bali, M., Covey, D. F., Evers, A. S., Steinbach, J. H., Zorumski, C. F., and Mennerick, S. (2007) *Pharmacol. Ther.* **116**, 35–57
29. Liao, M., Sonner, J. M., Husain, S. S., Miller, K. W., Jurd, R., Rudolph, U., and Eger, E. I., II (2005) *Anesth. Analg.* **101**, 131–135