# 2-(3-Methyl-3*H*-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate: A Derivative of the Stereoselective General Anesthetic Etomidate for Photolabeling Ligand-Gated Ion Channels

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To locate general anesthetic binding sites on ligand-gated ion channels, a diazirine derivative of the potent intravenous anesthetic, *R*-(+)-etomidate (2-ethyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate), has been synthesized and characterized. *R*-(+)-Azietomidate [2-(3-methyl-3*H*-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate] anesthetizes tadpoles with an $EC_{50}$ of 2.2 $\mu$M, identical to that of *R*-(+)-etomidate. At this concentration both agents equally enhanced GABA-induced currents and decreased binding of the caged-convulsant [$^{35}$S]TBPS to $GABA_A$ receptors. In all of the above actions *R*-(+)-azietomidate is about an order of magnitude more potent than *S*-(−)-azietomidate, an enantioselectivity comparable to etomidate's. *R*-(+)-Azietomidate also inhibits acetylcholine-induced currents in nicotinic acetylcholine receptors, with about twice the potency of the parent compound. [$^3$H]Azietomidate photoincorporated into *Torpedo* nicotinic acetylcholine receptor-rich membranes. Desensitization decreased photoincorporation into the $\delta$-subunit and increased that into the α-subunit. The latter increase was confined to a proteolytic fragment containing the first three transmembrane segments. Thus, *R*-(+)-azietomidate is a potent stereoselective general anesthetic and an effective photolabel.

## Introduction

One of the primary targets for general anesthetics is the ligand-gated superfamily of ion channels that includes the $GABA_A$, glycine, nicotinic acetylcholine, and $5HT_3$ receptors.[1,2] The first two are anion channels whose gating is enhanced by general anesthetics, and the second two are cation channels that are inhibited by all but the general anesthetics smallest by volume. Largely on the basis of work with the abundant nicotinic acetylcholine receptor (nAcChoR) from *Torpedo*, these receptors are thought to have five subunits arranged with pseudo-five-fold symmetry around a central pore.[3−5] Each subunit has a large N terminus and four transmembrane sequences (M1−4), with M2 lining the ion-conducting pathway. Kinetic[6,7] and direct binding studies[8] point to the existence of general anesthetic sites on the nAcChoR, and site-directed mutagenesis suggests this inhibitory site is associated with M2 amino acids lining the ion channel.[9,10] The enhancing site for volatile anesthetics on the $GABA_A$ receptors is associated with residues on the M1, M2, and M3 helices of the α-subunit that are hypothesized to surround a binding pocket for these agents.[2,11,12]

Etomidate is one of the most potent general anesthetics employed in clinical practice. The *R*-(+) enantiomer, which is the one used in the clinical formulation, is an order of magnitude more potent than the *S*-(−) enantiomer.[13] Although etomidate acts on other members of the ligand-gated ion channel superfamily, it is relatively selective for the $GABA_A$ receptor,[14] with which it interacts with enantioselectivity similar to that observed for general anesthesia.[13,15] The enhancing action of etomidate is highly dependent on the subtype of $\beta$-subunit. The higher sensitivity of the $\beta_2$- and $\beta_3$-subunits over the $\beta_1$-subunit is associated with a single residue within M2.[16,17] This residue corresponds to that within M2 on the α-subunit of the $GABA_A$ receptor associated with volatile general anesthetics action.[11] However, it remains an open question whether these amino acid residues on M1, M2, and M3 form a binding pocket, or whether they are simply involved in the transduction mechanism, allosterically altering gating in such a way as to modulate anesthetic action.[12,18−20]

In the absence of atomic resolution structures of these receptors, the above uncertainties cannot be resolved. An alternative approach to this issue is to employ photoaffinity labeling.[21] Recently, we introduced the use of diazirine-bearing general anesthetics for this purpose.[22] The diazirine moiety is minimally perturbing and can be introduced into a range of compounds. Our first ligand, 2-(3-pentyl-3*H*-diaziren-3-yl)ethanol (3-azioctanol), proved to be a good general anesthetic that photoincorporated into the M2 helix of nAcChoR's α-subunit.[22,23] Other researchers have since developed

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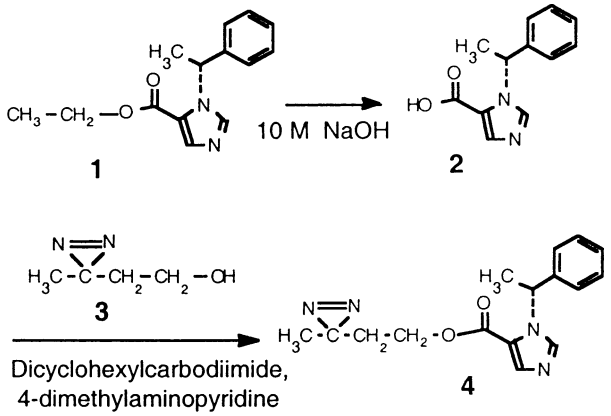
**Scheme 1**




diazirine derivatives of volatile anesthetics.[24] Here we introduce a diazirine derivative of the intravenous general anesthetic etomidate.

## Chemistry

Our aim was to synthesize a photoactivable analogue of the general anesthetic etomidate that both retains the anesthetic potency and stereospecificity of the parent compound and possesses a comparatively small photoreactive group with desirable photochemical properties. Scheme 1 shows the strategy for the synthesis of such a derivative, 2-(3-methyl-3*H*-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate (azietomidate, **4**). The starting material was 2-ethyl 1-(phenylethyl)-1*H*-imidazole-5-carboxylate (etomidate, **1**), which can be obtained in chirally pure forms. The ethyl ester linkages of *R*- and *S*-enantiomers of etomidate were cleaved by alkaline hydrolysis. The carboxyl group of the resulting product (**2**) was esterified with 2-(3-methyl-3*H*-diaziren-3-yl)ethanol (3-azibutanol, **3**), using dicyclohexylcarbodiimide and a nucleophilic catalyst, *p*-(dimethylamino)pyridine, to give **4**.

## Results

**General Anesthetic Potency.** The steep concentration−response curves for loss of righting reflexes (LORR) in *Xenopus* tadpoles are shown in Figure 1. *R*-(+)-Azietomidate's potency was identical to that of *R*-(+)-etomidate ($EC_{50}$ values of 2.2 ± 0.14 and 2.3 ± 0.13 $\mu$M, respectively). *S*-(−)-Azietomidate was more potent than *S*-(−)-etomidate, with an $EC_{50}$ of 14 ± 1.1 $\mu$M compared to 25 ± 2.2 $\mu$M. Our values for the *R*-(+)- and *S*-(−)-etomidate enantiomers are comparable to values of 3.4 ± 0.1 and 57 ± 1 $\mu$M, respectively, previously reported in *Rana temporaria* in propylene glycol solutions.[13] All animals fully recovered from exposure to the anesthetics.

The octanol/water partition coefficient of [$^3$H]azietomidate was found to be 3600 ± 210, and the saturated solubility of azietomidate in 0.05 M Tris-HCl buffer, pH 7.4, was 0.54 mM.

**Biphasic Potentiation of GABA-Activated Currents.** In its ability to enhance currents elicited by low concentrations of GABA, *R*-(+)-azietomidate resembled *R*-(+)-etomidate (Figure 2, top). Using GABA concentrations that elicited 5% of the maximum current ($EC_5$) from GABA receptors consisting of $\alpha_1\beta_2\gamma_{2L}$-subunits, both anesthetics enhanced responses at concentrations

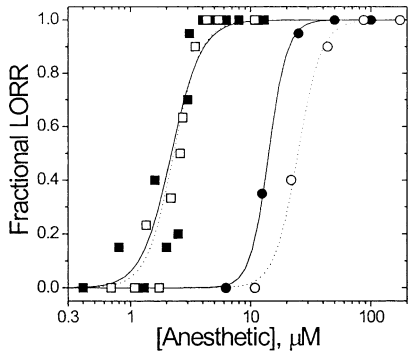



**Figure 1.** General anesthetic potency of azietomidate and etomidate enantiomers. *R*- (squares) and *S*-enantiomers (circles) of azietomidate (solid symbols) and etomidate (open symbols) were studied in *Xenopus* tadpoles. Animals were exposed to the anesthetic for 30−40 min before their loss of righting reflex (LORR) was checked. For clarity, at each concentration individual experiments were pooled to give groups of 10−20 tadpoles except for two points each that represent 5 and 30 tadpoles. The total number of animals, the $EC_{50}$ ($\mu$M), and the slopes of the dose response curves of the drugs investigated were as follows (errors are standard deviations, SD): *R*-(+)-etomidate, 170, 2.3 ± 0.13, and 3.9 ± 0.65; *R*-(+)-azietomidate, 190, 2.2 ± 0.14, and 3.5 ± 0.52; *S*-(−)-etomidate, 80, 25 ± 2.2, and 4.5 ± 1.0; *S*-(−)-azietomidate, 90, 14 ± 1.1, and 5.7 ± 1.5, respectively. Solid and dashed curves refer to the azietomidate and etomidate enantiomers, respectively.

above 0.1 $\mu$M. By 10−32 $\mu$M the enhanced currents reached magnitudes similar to those elicited by high concentrations of GABA alone. At higher concentrations (0.1−1.0 mM), an inhibitory process occurred that decreased the observed enhancement. Because these drugs display two opposing actions with overlapping concentration ranges, the data were fitted to a biphasic logistic expression (eq 2).[25] This analysis showed that *R*-(+)-azietomidate and -etomidate enhanced GABA-induced currents with $EC_{50}$ values that were equal to each other and to their general anesthetic $EC_{50}$ values (see caption to Figure 2). Their maximum levels of enhancement were similar and, for *R*-(+)etomidate, close to that previously reported for $GABA_A$ receptors of the same subunit composition ($EC_{50}$ = 1.2 ± 0.1 $\mu$M; maximum = 127 ± 12%).[16] Both of the *S*-(−) agents were an order of magnitude less potent than their *R*-(+) enantiomers and enhanced currents less effectively (see below).

Inhibition of enhanced currents was observed for all agents at or above 100 $\mu$M. The azietomidates were more potent than their corresponding etomidate enantiomers, with *R*-(+)-azietomidate being 5.5 times more potent as an inhibitor than *R*-(+)-etomidate. The weak enantioselectivity of inhibition was difficult to quantify because the concentration ranges over which enhancement and inhibition occurred overlapped more for the *S*-(−)- than the *R*-(+)-enantiomers and most of all for *S*-(−)-azietomidate, which caused only a modest apparent enhancement of some 30%. This limited our ability to analyze the data of the *S*-(−)-enantiomers, and their half-effect values are subject to systematic errors resulting from the need to fix maximum responses at an assumed value during curve fitting. We chose to constrain these fits by assuming that the efficacies of these compounds matched those of the corresponding *R*-enantiomers (see caption to Figure 2, top), a strategy that we have adopted previously.[26−28]

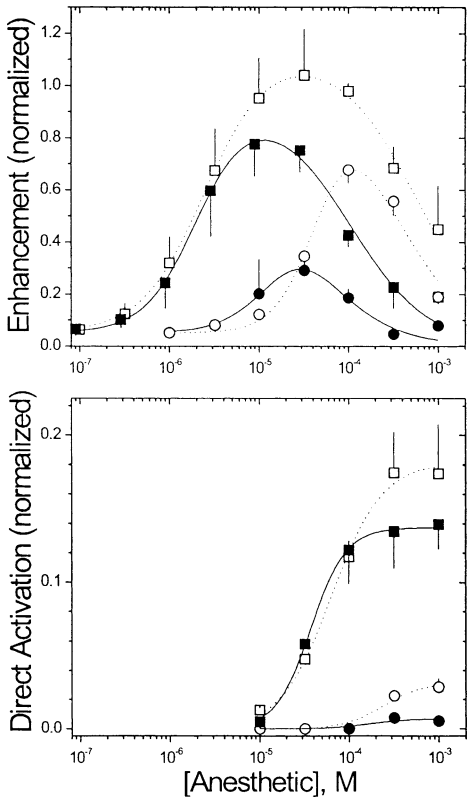



**Figure 2.** Action of azietomidate and etomidate enantiomers on $GABA_A$ receptors. Symbols and lines are the same as in Figure 1 [*R*- (squares) and *S*-enantiomers (circles) of azietomidate (solid symbols) and etomidate (open symbols)]. Bars are standard deviations. (Top) Currents elicited by GABA at $EC_5$ (6−12 $\mu$M) were enhanced by increasing concentrations of the coapplied anesthetics. Responses were normalized to the maximum responses that were elicited by a saturating concentration of GABA (1 mM). The curves are the result of fitting the data to a biphasic logistic expression (eq 2) accounting for enhancement at low concentrations and inhibition at high concentrations. For enhancement the parameters with their standard deviations are in the order $EC_{50}$ ($\mu$M), Hill coefficient, and maximum response. For the *R*-(+)-azietomidate and -etomidate these are 2.0 ± 0.51, 1.5 ± 0.38, 0.94 ± 0.11; and 2.5 ± 0.24, 1.2 ± 0.11, 1.1 ± 0.036, respectively. For the *S*-(−)-azietomidate and -etomidate these are 20 ± 5.0, 1.6 ± 0.36, 0.94 (fixed, see Results); and 47 ± 4.8, 1.9 ± 0.33, 1.1 (fixed, see Results), respectively. For inhibition the $IC_{50}$ values ± SD ($\mu$M) were in the order above 104 ± 32, 577 ± 66, 26 ± 6.1, and 295 ± 39. (Bottom) Currents elicited by the anesthetics in the absence of GABA. For technical reasons a two-step normalization protocol was applied as described under Experimental Section. Responses were first normalized to a standard concentration of the same drug (32 $\mu$M for *R*-enantiomers and 320 $\mu$M for *S*-enantiomers). The response of this standard concentration was then normalized to the response at 1 mM GABA alone. Next, the quotient of the first normalization step was multiplied by the corresponding quotient of the second normalization step to obtain responses of the four drugs that are normalized to 1 mM GABA. Data for the *R*-(+)-enantiomers were fitted to a logistic function by nonlinear least squares. For *R*-(+)-azietomidate and -etomidate the parameters (± SD), given in the order $EC_{50}$ ($\mu$M), Hill coefficient, and maximum response, are, respectively, 37 ± 1.5, 2.2 ± 0.20, 0.14 ± 0.002 and 64 ± 7.2, 1.5 ± 0.21, 0.18 ± 0.007.

**Direct Activation of $GABA_A$ Receptors.** All four agents were able to directly activate $GABA_A$ receptors in the absence of an agonist. *R*-(+)-Azietomidate and -etomidate directly activated currents at concentrations above 10 $\mu$M, with the former being some 2-fold more potent ($EC_{50}$ values of 37 ± 1.5 and 64 ± 7.2 $\mu$M, respectively; Figure 2, bottom). Neither was particularly efficacious, with maximum currents being 14 and 18% of those elicited by GABA alone. Our values for *R*-(+)-etomidate are close to those previously reported for GABA receptors of the same subunit composition ($EC_{50}$ = 83 ± 34 $\mu$M; maximum = 19 ± 2%).[16]

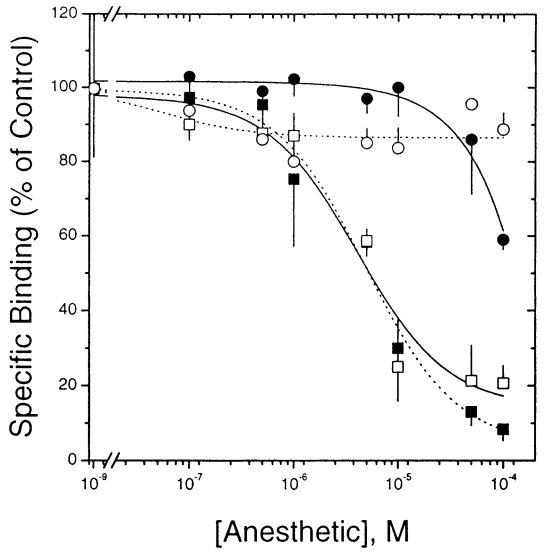



**Figure 3.** Enantiospecific inhibition by azietomidate and etomidate enantiomers of the specific binding of the cage convulsant [$^{35}$S]TBPS to $GABA_A$ receptors in total rat brain homogenates (mitochondrial plus microsomal fraction). Each point represents quadruplicate samples. Symbols and lines are as in Figure 1 [*R*- (squares) and *S*-enantiomers (circles) of azietomidate (solid symbols) and etomidate (open symbols)]. The *R*-(+)-azietomidate and -etomidate had $IC_{50}$ values of 4.2 ± 0.74 and 4.9 ± 0.90 $\mu$M, respectively. At 100 $\mu$M, *S*-(−)-etomidate had little effect, whereas *S*-(−)-azietomidate reduced specific binding to 60 ± 2.6% of control.

The *S*-(−)-enantiomers appeared to be both less potent and less efficacious. Currents were activated by *S*-(−)-etomidate at concentrations above 100 $\mu$M, but the maximal current was only 2.9 ± 0.58% of the 1 mM GABA control, rendering further analysis inappropriate. *S*-(−)-Azietomidate was a very weak direct activator, eliciting only about 0.5% of the maximal control GABA current at concentrations >320 $\mu$M.

Direct activation occurs over the same concentration range as inhibition of GABA-induced currents. Inhibition may simply be masked by activation, or these anesthetics may have some partial agonist character. These questions are not addressed herein.

**Allosteric Regulation of the $GABA_A$ Receptor.** *R*-(+)-Azietomidate inhibited specific [$^{35}$S]TBPS binding to total rat brain membranes at concentrations >0.5 $\mu$M, causing 80−90% inhibition at the highest concentration examined (100 $\mu$M; Figure 3). Analysis of the inhibition curve gave an $IC_{50}$ of 4.9 ± 0.90 $\mu$M, which was indistinguishable from that of *R*-(+)-etomidate. These actions were strongly enantioselective. Both *S*-(−)-enantiomers were without consistent effects at up to 50 $\mu$M, but *S*-(−)-azietomidate was more potent because at 100 $\mu$M it reduced specific binding to 60 ± 2.6% of control.

**Inhibition of the nAcChoR.** Effects of anesthetics were tested in the presence of 10 $\mu$M AcCho ($EC_{50} \approx 25$ $\mu$M for *Torpedo* and mouse nAcChoRs). Data were fitted

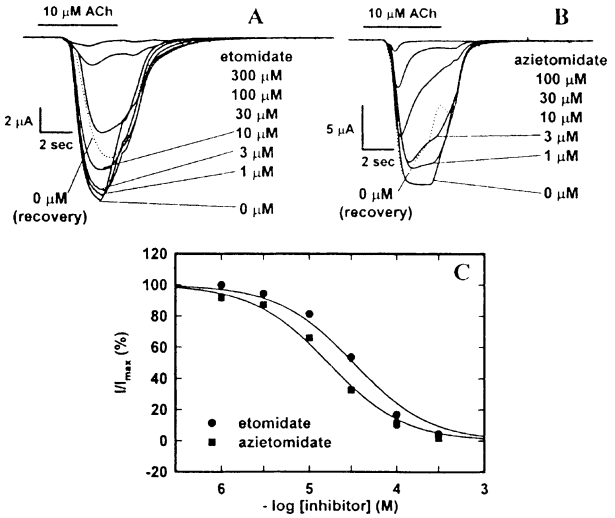



**Figure 4.** Azietomidate and etomidate inhibition of nicotinic acetylcholine receptor function. (A, B) Currents elicited by AcCho from *Torpedo* nAcChoRs expressed in *Xenopus* oocytes were measured by two electrode voltage clamps at −70 mV. Once a stable response was measured for AcCho (10 $\mu$M), responses were measured when AcCho was coapplied applied for 5 s with the indicated concentrations of etomidate (A) or azietomidate (B). The inhibition was at least 70% reversible, as indicated by the recovery of the AcCho responses after the oocytes were washed for 5 min following exposure to AcCho and 300 $\mu$M drug (dotted line). (C) Peak currents (I) elicited by AcCho in the presence of etomidate or azietomidate were normalized to the current in the absence of inhibitor. The data represent the mean ± SD of measurements made on one day from three oocytes.

to the equation $I_{Ach}^{Anes}I/I_{Ach}^{0} = [1 + ([Anes]/IC_{50})]^{-1}$, where $IC_{50}$ is the anesthetic concentration inhibiting half of the maximal current. When *R*-(+)-azietomidate was coapplied with acetylcholine to recombinant *Torpedo* nAcChoRs, it inhibited currents with an $IC_{50}$ of 23 ± 11 $\mu$M (data from six oocytes). For *R*-(+)-etomidate, the $IC_{50}$ was 41 ± 13 $\mu$M (Figure 4). For *R*-(+)-azietomidate in particular, at each concentration tested there was a time-dependent decrease of the AcCho response over the 5 s exposure. Further studies are required to determine whether azietomidate increases the rate of agonist-dependent desensitization or equilibrates only slowly with its binding site. The drugs had the same potencies when tested as inhibitors of recombinant embryonic mouse muscle nAcChoRs [azietomidate, $IC_{50}$ = 25 ± 5 $\mu$M; etomidate, $IC_{50}$ = 50 ± 7 $\mu$M (three oocytes)]. For each drug when assayed under these conditions the inhibition produced by 300 $\mu$M drug was reversed by at least 70% after a wash of 5 min. The *S*-(−)-enantiomers were not examined because strong stereoselectivity among channel inhibitors is rarely observed in nAcChoRs.

**Photoincorporation of [$^3$H]Azietomidate into the nAcChoR-Rich Membranes.** Initially, we compared the photoincorporation of [$^3$H]azietomidate and [$^3$H]etomidate at the same concentration and radiochemical specific activity. Tritium incorporation into polypeptides increased linearly during 6 min of irradiation at 254 nm, with no incorporation above background (40 cpm) being detected in the absence of irradiation. [$^3$H]Azietomidate proved to be by far the better photolabel, with levels of photoincorporation being >10 times those seen with [$^3$H]etomidate (data not shown). Irradiation with the 365 nm lamp resulted in photolabeling by [$^3$H]azietomidate of the same membrane polypeptides as at 254 nm with the same dependence upon addition of agonist or non-radioactive etomidate (data not shown).

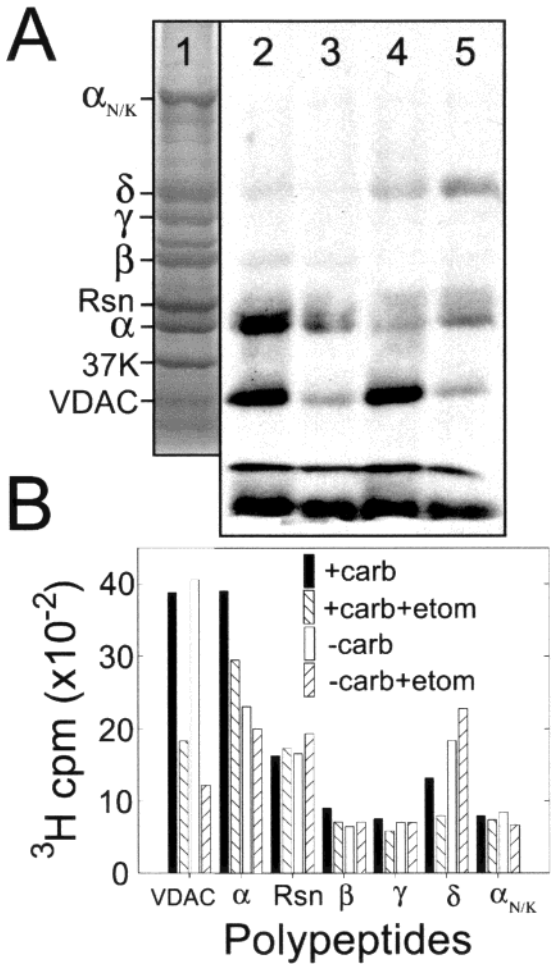



**Figure 5.** [$^3$H]Azietomidate photoincorporates into *Torpedo* nAcChoR. The nAcChoR-rich membranes were photolabeled with [$^3$H]azietomidate as described under Experimental Section. Membrane suspensions were equilibrated with 0.9 $\mu$M [$^3$H]azietomidate in the presence or absence of 300 $\mu$M carbamylcholine and/or 300 $\mu$M *R*-etomidate as indicated below. (A) Polypeptides were resolved by SDS−PAGE, visualized by Coomassie Blue stain (lane 1), and processed for fluorography (14-day exposure, lanes 2−5): lane 2, + carbamylcholine/− etomidate; lane 3, + carbamylcholine/+ etomidate; lane 4, − carbamylcholine/− etomidate; lane 5, − carbamylcholine/+ etomidate. Indicated on the left are the mobilities of the nAcChoR subunits (α, $\beta$, $\gamma$, $\delta$), rapsyn (Rsn), the α-subunit of the $Na^+/K^+$ATPase ($\alpha_{NK}$), and mitochondrial chloride channel (VDAC). (B) $^3$H incorporation into membrane polypeptides was quantified by scintillation counting of the gel bands excised from the gel after fluorography.

The pattern of photoincorporation of [$^3$H]azietomidate into nAcChoR-rich membranes isolated from *Torpedo* electric organ was examined by SDS−PAGE. The nAcChoR makes up ∼10% of the protein in this preparation. Other proteins are associated with the postsynaptic membrane, such as the peripheral protein rapsyn (43 kDa, present at equal abundance as nAcChoRs); contaminating membranes from the noninnervated surface of the electrocyte that are enriched in Na/K-ATPase (α-subunit ∼ 90 kDa); and membrane fragments from

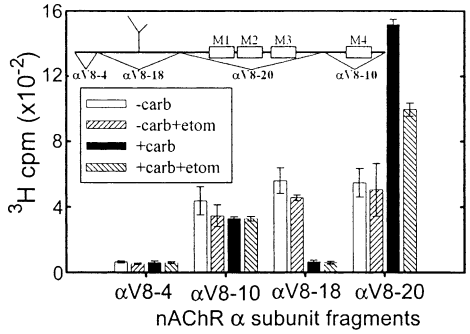



**Figure 6.** Mapping of [$^3$H]azietomidate photoincorporation into nAcChoR α-subunit by use of *S. aureus* V8 protease. Aliquots (500 $\mu$g) of nAcChoR-rich membranes were photolabeled with 0.9 $\mu$M [$^3$H]azietomidate. Aliquots were labeled in the absence or presence of 300 $\mu$M carbamylcholine and in the absence or presence of *R*-etomidate. Polypeptides were resolved by SDS−PAGE on an 8% gel as in Figure 5, and the α-subunit from each lane was excised and transferred to the wells of a 15% mapping gel for digestion with 5 $\mu$g of V8 protease as described under Experimental Section. The mapping gel was stained with Coomassie Blue to identify the four α-subunit fragments produced by V8 protease, and $^3$H incorporation in the fragments was determined by liquid scintillation counting. Bars indicate the mean ± SD of two samples. (Inset) Schematic showing the approximate α-subunit cleavage sites of *S. aureus* V8 protease. Fragments are identified by their molecular weights in kDa as follows: αV8-4 begins at αSer-1; αV8-18 begins at αVal-46 and contains AcCho binding site segments A (αTyr-93) and B (αTrp-149); αV8-20 begins at αSer-173 and contains segment C of the AcCho binding site (αTyr-190 to αTyr-198) as well as M1, M2, and M3; αV8-10 runs from αAsn-339 to the C terminus including the highly lipid-exposed M4.

mitochondria enriched in the 34 kDa voltage-dependent anion channel (VDAC).[29] Under the conditions described in Figure 5, for membranes labeled in the presence of carbamylcholine, the major sites of $^3$H incorporation were within the nAcChoR α-subunit and VDAC. Photoincorporation within the nAcChoR subunits depended upon the conformational state of the receptor, with that in the α-subunit being enhanced and that in the $\delta$-subunit being decreased by the addition of carbamylcholine. Photoincorporation into the $\beta$- and $\gamma$-subunits was not regulated by the ligands added and occurred at only the same low level as that into the α-subunit of Na/K-ATPase. Photoincorporation into nonreceptor polypeptides was also insensitive to carbamylcholine. In the presence of carbamylcholine, 300 $\mu$M *R*-(+)-etomidate reduced photoincorporation of [$^3$H]azietomidate into the α- and $\delta$-subunits of the nAcChoR. It also did so in VDAC but independent of the presence of carbamylcholine.

To further localize the sites of [$^3$H]azietomidate incorporation within the nAcChoR α-subunit, *Staphylococcus aureus* V8 protease was used to fragment the labeled α-subunits into four large, non-overlapping polypeptides resolvable by SDS−PAGE.[30] In the absence of carbamylcholine, $^3$H incorporation was evenly distributed among αV8-18, αV8-20, and αV8-10 (Figure 6). In contrast, after photolabeling in the presence of carbamylcholine, 75% of $^3$H was within αV8-20, 15% within αV8-10, and <5% in αV8-18 or αV8-4. The presence of carbamylcholine had no effect on the $^3$H incorporation into αV8-10 or αV8-4, but it resulted in a 2.5-fold increase in the labeling within αV8-20 and a 90% reduction in the incorporation within αV8-18. In the absence of carbamylcholine, non-radioactive etomidate had little effect on the $^3$H photoincorporation, whereas in the presence of carbamylcholine etomidate strongly reduced incorporation but only into αV8-20.

## Discussion

**General Anesthetic Properties.** The tadpole is a traditional animal for measuring in vivo general anesthetic potency when limited quantities of material are available. *R*-(+)-Azietomidate behaved as a typical reversible nontoxic general anesthetic, causing LORR in tadpoles with high potency identical to that of its unmodified enantiomer. The potency of a general anesthetic can be predicted with good accuracy by the empirical Meyer−Overton rule, which states that the product of the anesthetic $EC_{50}$ and the octanol/water partition coefficient is constant. *S*-(−)-Azietomidate fits this rule well, but *R*-(+)-azietomidate is significantly more potent than expected.

**Actions on the $GABA_A$ Receptor.** Although it is not the only target, a strong case has been made that general anesthetic−$GABA_A$ receptor interactions play a primary role in the induction of the state of general anesthesia.[1,2,31,32] At pharmacological concentrations general anesthetics enhance inhibitory currents induced by subsaturating concentrations of GABA. *R*-(+)- and *S*-(−)-azietomidate behaved like other general anesthetics, causing half-maximal enhancement at their respective general anesthetic $EC_{50}$ values.

At higher concentrations all four agents inhibited these enhanced GABA-induced currents. The azietomidates were more potent inhibitors than the etomidates, so that in their case the net enhancement of GABA-induced currents at anesthetic concentrations was limited by inhibition. Kinetic and site-directed mutagenesis experiments suggest that the site underlying this inhibitory action is independent of that underlying enhancement.[33,34] Our observations support this conclusion because the enantioselectivity that occurred for enhancement was reversed for inhibition.

As previously reported, at high concentrations *R*-(+)-etomidate can directly and enantioselectively activate $GABA_A$ receptors by an unknown mechanism.[13,15,35] Our work shows that the azietomidate enantiomers behave similarly (Figure 2, top).

The binding of the picrotoxinin-like cage convulsant TBPS to $GABA_A$ receptors in mammalian brain membrane homogenates is known to be inhibited by many general anesthetics, including etomidate, which acts with enantioselectivity.[31,36,37] In this respect, *R*-(+) and *S*-(−)-azietomidate were almost identical to the etomidate enantiomers (Figure 3). At general anesthetic concentrations both *R*-(+)-enantiomers inhibited specific binding of [$^{35}$S]TBPS, whereas their *S*-(−)-enantiomers had little or no effect.

Thus, *R*-(+)-azietomidate closely resembles *R*-(+)-etomidate in all of the different physiological and biochemical interactions with $GABA_A$ receptors investigated above, suggesting that it will be a most useful photolabel with the potential to characterize each of the sites underlying its diverse pharmacological actions.

**Enantioselectivity of Action on $GABA_A$ Receptors.** The etomidate enantiomers exhibit an enantiospecificity that is much larger than that of any other

common general anesthetic.[13] This enantiospecificity is largely preserved in the azietomidate enantiomers, the ratios of $EC_{50}$ values [*S*(−)/*R*(+)] (± SD) being 6.4 ± 0.65 and 11 ± 1.2 for the azietomidate and etomidate enantiomers, respectively (Figure 1). These ratios compare to somewhat larger values for enhancement of GABA currents (Figure 2, top) of 12 ± 4.5 and 19 ± 2.6, respectively, suggesting that the particular subtype of $GABA_A$ receptor studied here is not the sole site of general anesthetic action in vivo. Furthermore, inhibition of [$^{35}$S]TBPS binding was also strongly enantioselective with both *S*-(−) enantiomers having $IC_{50}$ values >100 $\mu$M (enantioselectivities >20-fold). This enantioselectivity will offer an additional advantage in the assessment of the pharmacological role of sites of photoincorporation revealed in future research.

**Photoincorporation into the nAcChoR**. The $GABA_A$ receptor has such low abundance in brain that determining which amino acid residues are photolabeled presents a formidable challenge that is beyond the scope of this paper.[38,39] In contrast, the abundant *Torpedo* nAcChoR has been the subject of many detailed photolabeling studies, the results of which have proved to be useful when extrapolated to the other members of the superfamily.[4] Thus, examining [$^3$H]azietomidate's ability to photolabel the nAcChoR is an important step in assessing its utility as a photoaffinity reagent.

[$^3$H]Azietomidate was successfully photoincorporated into the nAcChoR, with the α- and δ-subunits being labeled preferentially. Two observations at the subunit level suggested some of this photoincorporation was at specific functional sites. First, it was allosterically regulated; conversion of the nAcChoR to the desensitized state by the addition of the agonist carbamylcholine doubled photoincorporation in the α-subunit and reduced that in the δ-subunit. Second, *R*-(+)-etomidate inhibited [$^3$H]azietomidate photoincorporation in the desensitized state.

At least three domains of the nAcChoR's α-subunit are photolabeled by [$^3$H]azietomidate (Figure 6), and two of them contain specific sites. The first is αV8-20, which contains the M2 ion channel domain as well as M1 and M3, where the enhanced labeling seen in the desensitized state is located. Second is αV8-18, which contains the extracellular domain of the nAcChoR N-terminal to M1, including the agonist site, where photoincorporation was inhibited by carbamylcholine, suggesting that residues within the AcCho binding site were labeled. Third is αV8-10, which contains the M4 hydrophobic segment that presents a large lipid−protein interface,[40] where photoincorporation was not modulated by added ligands, suggesting nonspecific photolabeling by [$^3$H]-azietomidate concentrated in the adjacent lipid bilayer.

The preferential labeling of nAcChoR's α-subunit in the desensitized state by [$^3$H]azietomidate resembles that previously observed with the general anesthetic [$^3$H]-3-azioctanol.[22,23] That agent also photolabeled the three proteolytic fragments identified above. Subsequent exhaustive studies identified amino acid residues that were photolabeled in each region, with the major site being αGlu-262.[23] This residue is at the extracellular C-terminal end of the M2 hydrophobic segment and, if this region is helical, it would be correctly oriented to contribute to the lumen of the ion channel.[4,5] Such studies for [$^3$H]azietomidate are now clearly feasible but beyond the scope of the work reported here.

**Conclusion.** We have synthesized and characterized the pharmacology of *R*-(+)-azietomidate, a photoactivable analogue of the intravenous general anesthetic *R*-(+)-etomidate. Replacement of an ethyl group by a 2-(3-methyl-3*H*-diaziren-3-yl)ethyl group caused only minor changes in the pharmacology and allowed the agent to be successfully photoincorporated into the nAcChoR, one member of a ligand-gated ion channel of the superfamily thought to underlie general anesthetic action. *R*-(+)-Azietomidate is some 2 orders of magnitude more potent than other diazirine-containing general anesthetics reported to date,[22,24] and it is enantioselective; both properties make it an advantageous ligand for characterizing general anesthetic binding sites.

## Experimental Section

**Abbreviations.** AcCho, acetylcholine; 3-azibutanol, 2-(3-methyl-3*H*-diaziren-3-yl)ethanol; azietomidate, 2-(3-methyl-3*H*-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate; 3-azioctanol, 2-(3-pentyl-3*H*-diaziren-3-yl) ethanol; etomidate, 2-ethyl 1-(phenylethyl)-1*H*-imidazole-5-carboxylate; LORR, loss of righting reflexes; nAcChoR, nicotinic acetylcholine receptor; octanol, octan-1-ol; SD, standard deviation; TBPS, *tert*-butyl-bicyclophosphorothionate; TPS, *Torpedo* physiological saline.

**Materials and Methods.** 3-Azibutanol was synthesized as described by Church and Weiss.[41] Anhydrous dichloromethane, dicyclohexylcarbodiimide, *p*-(dimethylamino)pyridine, and Merck silica gel 60 A, 230−400 mesh, were obtained from Aldrich (Milwaukee, WI). *R*-(+)- and *S*-(−)-etomidate were from Organon Laboratories, Newhouse, Lanarkshire, Scotland. *R*-(+)-Etomidate used in $GABA_A$ receptor electrophysiology studies was purchased from Bedford Laboratories (Bedford, OH) as a 2.0 mg/mL solution in 35% propylene glycol/water (v/v). All other chemicals were from Sigma (St. Louis, MO). cDNAs for the $\alpha_1$−, $\beta_2$-, and $\gamma_{2L}$-subunits of human $GABA_A$ receptors in pCDM8 vectors were gifts from Dr. Paul J. Whiting (Merck Sharp & Dohme Research Laboratories, Essex, U.K.).

$^1$H NMR spectra were recorded on a Bruker 400 MHz spectrometer in $CDCl_3$ with tetramethylsilane as reference. Mass spectrometry was performed on a Finnigan LCQ Deca with electrospray ionization. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN. UV spectra were recorded on a Hewlett-Packard spectrophotometer. HPLC analysis was performed on a Varian Prostar instrument.

**Preparation of 1-(1-Phenylethyl)-1*H*-imidazole-5-carboxylic Acid (2).** *R*-2-Ethyl-1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate (etomidate, **1**) was treated with sodium hydroxide to hydrolyze the ester bond essentially as described.[42] A mixture of *R*-etomidate (195 mg, 0.8 mmol) and 10 M NaOH (0.4 mL) was stirred in a sealed hydrolysis tube at 100 °C for 1 h. The reaction mixture was cooled, diluted with 0.4 mL of $H_2O$, and neutralized with 1 M HCl (4 mL). After rotary evaporation, the mixture was dried under vacuum. Analytical silica gel TLC showed that etomidate was completely hydrolyzed. The product, 1-(1-phenylethyl)-1*H*-imidazole-5-carboxylic acid, **2**, was used in the subsequent step without further purification.

**Preparation of *R*-2-(3-Methyl-3*H*-diaziren-3-yl)ethyl 1-(1-Phenylethyl)-1*H*-imidazole-5-carboxylate (*R*-azietomidate, 4).** To a suspension of **2** (0.8 mmol) in anhydrous dichloromethane (3.5 mL) was added **3** (160 mg, 1.6 mmol), *p*-(dimethylamino)pyridine (119 mg, 1.0 mmol), and dicyclohexylcarbodiimide (1.6 mL, 1 M solution in anhydrous dicholoromethane). The mixture was stirred for 15 h at room temperature. After removal of the precipitated dicyclohexylurea, the soluble product was applied to a column of silica gel (50 g) equilibrated with dichloromethane. Sequential

elution with dichloromethane and dichloromethane containing 5% ether gave a peak containing unreacted **3** ($\lambda_{max}$ 349 nm) followed by another peak containing the product **4** ($\lambda_{max}$ 345 and 242 nm). The latter fractions were rotary evaporated, and the residue was dissolved in ethyl acetate (10 mL) and cooled on ice. A small amount of dicyclohexylurea contaminant that crystallized from the solution was removed by filtration. The solution was evaporated to yield an oily residue of **4** (182 mg, 76% yield). The product was taken up in ether (25 mL), treated with 12.1 N HCl (60 $\mu$L), and cooled at −20 °C for 2 h to give white crystals of azietomidate hydrochloride (110 mg, 41% yield): NMR ($CDCl_3$) $\delta$ 1.09 (s, 3H), 1.75 (s, 2H), 1.85 (t, 3H, $J$ = 7.2 Hz), 4.27 (m, 2H), 6.51 (q, 1H, $J$ = 7.2 Hz), 7.37 (m, 2H), 7.44 (m, 3H), 8.06 (s, 1H), 8.87 (s, 1H); UV spectrum (methanol) $\lambda_{max}$ 345 nm, $\epsilon$ = 63 $M^{-1}$ $cm^{-1}$, $\lambda_{max}$ 241 nm, $\epsilon$ = 13700 $M^{-1}$ $cm^{-1}$ (equivalent figures determined for etomidate are 0 and 12200 $M^{-1}$ $cm^{-1}$, respectively). Elemental Anal. Calcd for $C_{16}H_{19}N_4O_2Cl$: C, 57.41; H, 5.71; N, 16.73; Cl, 10.59. Found: C, 56.49; H, 5.90; N, 16.30; Cl, 10.80

**Preparation of *S*-2-(3-Methyl-3*H*-diaziren-3-yl)ethyl 1-(1-Phenylethyl)-1*H*-imidazole-5-carboxylate (*S*-Azietomidate).** This enantiomer was synthesized starting with *S*-etomidate according to a procedure similar to the one described for the synthesis of **4**.

**Preparation of [$^3$H]-2-(3-Methyl-3*H*-diaziren-3-yl)ethyl 1-(1-Phenylethyl)-1*H*-imidazole-5-carboxylate ([$^3$H]Azietomidate).** *R*-(+)-Etomidate was custom tritiated at Perkin-Elmer Life Sciences, Boston, MA, by catalytic exchange reaction to give [*ring*-$^3$H(N)]-etomidate with a specific activity of 17 Ci/mmol. The mass spectrum fragmentation pattern of [$^3$H]etomidate showed that all of the tritium was in the imidazole ring, suggesting that the chirality was preserved during tritiation.

[$^3$H]etomidate (probably the *R*-enantiomer, see above; 0.725 micromol, 14.5 mCi) was treated with 10 M NaOH (0.1 mL) at 100 °C for 1 h to hydrolyze the ester bond. The product, [$^3$H]1-(1-phenylethyl)-1*H*-imidazole-5-carboxylic acid, was mixed with anhydrous dichloromethane (0.15 mL), 3-(2-hydroxyethyl)-3-methyl-3*H*-diazirine (2.5 $\mu$L), *p*-(dimethylamino)pyridine (1.85 mg), and dicyclohexylcarbodiimide (2.5 $\mu$L, 1 M solution in anhydrous dichloromethane). After 15 h of stirring at room temperature, the soluble product was applied to a column of silica gel (3.5 g) equilibrated with dichloromethane. Sequential application of dichloromethane and dichloromethane containing 5% ether eluted [$^3$H]azietomidate.

The tritiated product and unlabeled **4** eluted in the same position on HPLC and had the same $R_f$ on TLC. The specific activity of [$^3$H]azietomidate, determined by HPLC analysis, was 11.4 ± 0.28 Ci/mmol.

**General Anesthetic Potency.** With institutional approval, general anesthetic potency was assessed in pre-limb bud *Xenopus* tadpoles, 1.5−2 cm in length (Xenopus 1, Inc., Dexter, MI). Groups of 5−10 tadpoles were placed in covered 100 mL glass beakers or square slide-staining dishes in oxygenated aqueous solutions buffered with 5 mM Tris-HCl at pH 7.4 under low levels of ambient light. Agents were usually added from stock buffer solutions but in a few cases were added from stock solutions in ethanol. The final concentration of ethanol did not exceed 5 mM, a concentration that does not contribute to anesthesia.[43] Both methods gave identical results. Tadpoles were tipped manually with a flame-polished pipet, and failure to right after 5 s was defined as LORR. The response stabilized within 20 min, and measurements were made at 30−40 min. All animals were placed in fresh water and observed the next day for toxicity. The quantal concentration response curves were analyzed according to the method of Waud[44] using an Excel macro kindly provided by N. L. Harrison, A. Jenkins, and S. P. Singh (Weill Medical College of Cornell University).

**Solubility Properties.** The solubility in 0.05 M Tris-HCl buffer, pH 7.4, was determined by shaking with excess azietomidate for 3 h, centrifuging at 10000*g* for 3 min, and determining the concentration at 350 nm. The octanol/water partition coefficient was determined by shaking a two-phase mixture in an amber vial and allowing it to settle for 12 h before aliquots from each phase were withdrawn for analysis on an HPLC [reverse phase C-18 column (Varian, Walnut Creek, CA); water/acetonitrile gradient at 1 mL/min; detection at 241 nm].

**Electrophysiology of GABA and nAcChoR Receptors.** Recombinant wild-type $GABA_A$ receptors consisting of $\alpha_1\beta_2\gamma_{2L}$ subunits or wild-type *Torpedo* or embryonic mouse nAcChoRs consisting of $\alpha\beta\gamma\delta$-subunits were expressed in *Xenopus laevis* oocytes and studied by two electrode voltage clamps.

Capped mRNAs were transcribed in vitro from linearized cDNA templates and stored at −80 °C. Methods of oocyte preparation and injection have been described previously.[25,45] Defolliculated stage V and VI oocytes were injected with a 25−50 nL mixture of receptor subunit mRNAs, incubated at 17 °C, and used for electrophysiological experiments for up to 7 days.

Electrophysiology solutions were in ND-96 (96 mM NaCl, 2 mM KCl, 0.8 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 5−10 mM HEPES, pH 7.6). For $GABA_A$ receptor studies, *R*-(+)-etomidate solutions of up to 320 $\mu$M were prepared by diluting commercial stock (8.2 mM) into ND96. Solutions of higher concentration were prepared from 26.2 mM stocks in 35% propylene glycol/water. Solutions containing *S*-(−)-etomidate and isomers of azietomidate were made using stock solutions of up to 26.2 mM in 35% propylene glycol/water. The maximum concentration of propylene glycol in superfusate was 170 mM. Solutions for nAcChoR electrophysiology were low $Ca^{2+}$ ND-96 (0.3 mM $CaCl_2$) without propylene glycol.

Two microelectrode physiology methods have been previously described.[46] Experiments were performed at room temperature (20−22 °C). Oocytes were voltage clamped at −50 mV ($GABA_A$ receptors) or −70 mV (nAcChoRs). Gravity-driven superfusion solutions were controlled with computer-activated solenoid valves. Oocyte currents were elicited by agonist/etomidate solution exposures between 5 and 50 s, digitized at 50−200 Hz, recorded on a personal computer using commercial software (Clampex7; Axon Instruments, Inc., Foster City, CA), and analyzed off-line using Origin v 5.0 (Microcal Software, Inc., Northampton, MA).

**Direct Activation of $GABA_A$ Receptor Currents by Anesthetics.** Enantiomers of etomidate or azietomidate were applied for up to 60 s to stimulate whole oocyte currents ($n \geq 5$). No currents were directly evoked by propylene glycol alone at up to 170 mM. Peak currents at different anesthetic concentrations in each oocyte were normalized by pairing each response with a standard response elicited with either 32 $\mu$M for the *R*-enantiomers or 320 $\mu$M for the *S*-enantiomers. Washout times of up to 15 min were needed to reverse activation at concentrations >100 $\mu$M. Efficacy relative to GABA was estimated by experiments in separate oocytes ($n \geq 5$) that assessed relative currents evoked by 1 mM GABA and the relevant standard etomidate or azietomidate concentration. Normalized anesthetic concentration−response data were averaged and renormalized using the average response ratio for the standard anesthetic concentration and 1 mM GABA. Data were plotted as mean ± SD. Logistic (Hill) equations (eq 1) were fitted to concentration−response data to derive apparent efficacy ($I_{Anes}^{Max}/I_{GABA}^{Max}$), the half-maximal activating concentration ($EC_{50}$), and the Hill coefficient ($n$).

$$\frac{I_{Anes}}{I_{GABA}^{Max}} = \frac{I_{Anes}^{Max}}{I_{GABA}^{Max}} \times \frac{[Anes]^n}{[Anes]^n + EC_{50}{}^n} \quad (1)$$

**Potentiation of GABA-Evoked Currents by Anesthetics.** Enhancement of the $EC_5$ GABA response by enantiomers of etomidate or azietomidate was studied in whole oocytes. The concentration of GABA that evoked 5 ± 1% of the maximal GABA-activated current (at 1−10 mM) was determined for each oocyte (range = 6−12 $\mu$M). Oocyte currents evoked using coapplied GABA $EC_5$ plus anesthetic were paired with control currents elicited with 1 mM GABA alone with washout times of 10 min between experiments. Normalized oocyte responses ($n \geq 5$) were averaged. Biphasic concentration−response data

were plotted as mean ± SD and fitted to a biphasic logistic equation (eq 2) to derive apparent efficacy ($I^{Max}/I^{Max}_{GABA}$), half-maximal enhancing concentration ($C_{50}$) and its Hill coefficient ($n$), and half-maximal inhibiting concentration ($IC_{50}$).

$$\frac{I}{I^{Max}_{GABA}} = \left[0.05 + \left(\frac{I^{Max}}{I^{Max}_{GABA}} - 0.05\right) \times \frac{[Anes]^n}{[Anes]^n + {C_{50}}^n}\right] \times \frac{IC_{50}}{[Anes] + IC_{50}} \quad (2)$$

**Allosteric Regulation of the $GABA_A$ Receptor.** The binding of the allosteric $GABA_A$ receptor ligand *tert*-butyl [$^{35}$S]-bicyclophosphorothionate (TBPS) was measured as previously described,[47−49] using thoroughly washed total rat brain homogenates (mitochondrial plus microsomal fraction). Osmotically shocked, frozen and thawed membranes were equilibrated with ∼0.5 mg/mL protein in 0.1 M KCl, 10 mM potassium phosphate buffer, and 0.1 $\mu$M GABA, pH 7.5 at 4 °C. Incubation with [$^{35}$S]TBPS (6 nM; ∼100 Ci/mmol, Perkin-Elmer Life Sciences, Boston, MA; corrected specific radioactivity was calculated on the day of assay) was carried out at 21 °C for 90 min. A duplicate set of assay tubes ($n$ = 4) contained 10 $\mu$M picrotoxinin (Sigma, St. Louis, MO) was used to determine nondisplaceable binding, which was subtracted from total binding to calculate specific binding. After equilibration, the samples were vacuum filtered through Whatman GF/B filters using a Brandel Cell Harvester (Gaithersburg, MD) and counted in Cytoscint−toluene scintillation cocktail (ICN, Irvine, CA). Specific binding was >90% of the total.

The shape and potency of concentration-dependent modulation of binding curves varies with brain region (probably due to receptor subunit composition) and membrane preparation (possibly due to endogenous GABA and other substances such as steroids). The variability can be minimized by including a low concentration (≤1 $\mu$M) of GABA in the assays in vitro.[47,49,50] Therefore, 0.1 $\mu$M GABA was included in all [$^{35}$S]TBPS binding assays employed herein.

**Photoincorporation of [$^3$H]Azietomidate into the nAcChoR.** nAcChoR-rich membranes, prepared as described[45] from *Torpedo californica* electric organ, were suspended at a final protein concentration of 2 mg/mL in *Torpedo* physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0) supplemented with 1 mM oxidized glutathione; 100 $\mu$L aliquots of membrane suspensions were incubated with 0.9 $\mu$M [$^3$H]azietomidate in the presence or absence of ligands, and the samples were irradiated at 4 °C at a distance of 6 cm with either a 254 nm lamp (Spectroline EF-16, Spectronics Corp., Westbury, NY; Hg line) or a 365 nm lamp (Spectroline EN-16), which has a broad emission band centered at 352 nm (±19 nm at half-height) superimposed on the 365 nm Hg line and no intensity below 300 nm. On the basis of previous experience with photoincorporation of aliphatic diazirines into nAcChoR-rich membranes using these lamps,[23] samples were irradiated for 6 min at 254 nm or for 25 min at 365 nm.

Electrophoresis sample-loading buffer was added to the samples, and they were subjected to SDS−PAGE on 1.5 mm thick 8% polyacrylamide gels with 0.33% bis(acrylamide). Polypeptides were visualized by staining with Coomassie Blue R-250 (0.25% w/v in 45% methanol and 10% acetic acid) and destained in 25% methanol and 10% acetic acid. The gels were then impregnated with fluor (Amplify, Amersham Biosciences, Piscataway, NJ) for 20 min with shaking, dried, and exposed to film (Kodak X-Omat Blue XB-1, Eastman Kodak, Rochester, NY) for 2 weeks. Incorporation of $^3$H into individual polypeptides excised from the stained gels was determined by liquid scintillation counting.[22] Proteolytic digestion of the isolated α-subunit with *S. aureus* V8 protease was performed in gel as described.[30] Photolabeling was carried out with 500 $\mu$g aliquots of nAcChoR membranes and 0.9 $\mu$M [$^3$H]azietomidate. The α-subunits were excised from the first gel and transferred to a 15% "mapping gel" for digestion with V8 protease. The mapping gel was then stained, destained, and cut into strips, and $^3$H incorporation into the α-subunit fragments was determined by liquid scintillation counting.

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