0026-895X/06/6906-2015–2026$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:2015–2026, 2006

Vol. 69, No. 6
22590/3117916
Printed in U.S.A.

Dual Potentiating and Inhibitory Actions of a Benz[e]indene Neurosteroid Analog on Recombinant $\alpha1\beta2\gamma2$ $GABA_A$ Receptors

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Received January 16, 2006; accepted March 22, 2006

ABSTRACT

Benz[e]indenes are tricyclic analogs of neuroactive steroids and can be modulators of $GABA_A$ receptor activity. We have examined the mechanisms of action of the benz[e]indene compound [3*S*-(3α,3aα,5aβ,7β,9aα,9bβ)]-dodecahydro-7-(2-hydroxyethyl)-3a-methyl-1*H*-benz[e]indene-3-carbonitrile (BI-2) using single-channel patch-clamp and whole-cell recordings from human embryonic kidney cells transfected with rat $GABA_A$ receptor $\alpha1$, $\beta2$, and $\gamma2$L subunits. The data demonstrate that BI-2 is a positive modulator of $GABA_A$ receptor activity with a peak effect at 2 μM. The mechanism of modulation is similar but not identical to that of neuroactive steroids. Similar to steroids, BI-2 acts by prolonging the mean open time duration through an effect on the duration and prevalence of the longest open time component. However, in contrast to many steroids, BI-2 does not selectively reduce the channel closing rate. The potentiating action of BI-2 seems to be mediated through interactions with the classic neuroactive steroid binding site. Mutation to the membrane-spanning region in the $\alpha1$ subunit Q242W and the double mutation $\alpha1$N408A/Y411F, previously shown to abolish potentiation by neurosteroids, also diminish potentiation by BI-2. At higher concentrations (>5 μM), BI-2 inhibits receptor function by enhancing the apparent rate of desensitization. From single-channel recordings, we estimate that the entry rate into the inhibited or blocked state, k_{+B}, is 0.50 μM^{-1} s^{-1}. Based on the kinetic mechanism of action, and the finding that this effect is blocked by the $\alpha1$V256S mutation, we propose that BI-2 acts through an inhibitory site first postulated for the inhibitory neurosteroid pregnenolone sulfate.

Most of fast synaptic inhibition in the central nervous system is accomplished through the activation of $GABA_A$ receptors. The $GABA_A$ receptors are permeable to Cl^-, so channel opening results in hyperpolarization of the cell or dampening of depolarizing actions of excitatory neurotransmitter receptors. The channel activity can be enhanced or inhibited by a variety of compounds, such as benzodiazepines, neuroactive steroids, and barbiturates. Many of these compounds are in clinical use where they are used as anxiolytics, anticonvulsants, or anesthetics.

Benz[e]indenes are tricyclic analogs of steroids in which the B, C, and D rings are retained intact but which contain only a portion of the A ring (Fig. 1). This results in structurally flexible compounds that can adapt multiple conformations.

Previous work has shown that benz[e]indenes potentiate GABA-activated currents in hippocampal neurons (Rodgers-Neame et al., 1992; Hu et al., 1993). Because of the structural similarity of these tricyclic compounds and steroids, it was hypothesized that benz[e]indenes act on the $GABA_A$ receptor through molecular interactions with the steroid binding site (Rodgers-Neame et al., 1992). Several lines of evidence supported this hypothesis. First, it was shown that antagonists to the benzodiazepine (flumazenil) and picrotoxin sites (α-isopropyl-α-methyl-γ-butyrolactone) did not block the ability of a benz[e]indene to potentiate GABA currents. Second, it was shown that the ability of benz[e]indenes to potentiate $GABA_A$ receptor function is greatly inhibited in the presence of the neurosteroid (3α,5α)-3-hydroxypregnan-20-one (allopregnanolone). Conversely, the presence of benz[e]indene diminished the ability of allopregnanolone to further potentiate receptor function. However, it may be argued that the presence of high concentrations of steroid results in a maximal possible channel open probability so that no further potentiation is possible whether the two drugs act through

J.H.S. is the Russell and Mary Shelden Professor of Anesthesiology. This work was supported by National Institutes of Health grants GM47969 and AA14707.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.022590.

ABBREVIATIONS: BI-2, [3*S*-(3α,3aα,5aβ,7β,9aα,9bβ)]-dodecahydro-7-(2-hydroxyethyl)-3a-methyl-1*H*-benz[e]indene-3-carbonitrile; HEK, human embryonic kidney; OT, open time; B285, (3α,5β,17β)-3-hydroxy-18-norandrostane-17-carbonitrile; CT, closed time.

the same site or not. Indeed, our previous single-channel work with neuroactive steroids suggests that the burst open probability, a parameter that is the main determinant of macroscopic peak current, is close to one in the presence of high concentrations of steroids (Akk et al., 2004).

Although previous electrophysiological work provides some information on how benz[*e*]indenes modulate $GABA_A$ receptor function, a more complete characterization of their modes and sites of action is warranted. For example, previous work from this laboratory and others has suggested that steroid interactions with the channel are mediated through, at least, two distinct sites (Twyman and Macdonald, 1992; Akk et al., 2004). It is not known whether benz[*e*]indenes, which lack part of the fixed steroid backbone, are able to successfully dock in both sites and cause the structural reorientation that must precede the kinetic effect. In addition, the possibility that benz[*e*]indenes have unique actions that differ from those of steroids has not been addressed.

In the present work, we have applied single-channel kinetic analysis and whole-cell voltage-clamp to examine the mechanisms of action of the benz[*e*]indene compound BI-2 (Fig. 1). The data demonstrate that BI-2 has dual actions on $GABA_A$ channel function. At low concentrations, the predominant effect is one of potentiation, mediated by prolongation of channel openings. From a mechanistic standpoint, the effect is similar to that observed in the presence of neuroactive steroids: the long-lived openings become longer and more prevalent as the BI-2 concentration is raised. However, in contrast to, at least, some neuroactive steroids (Akk et al., 2004, 2005), the presence of BI-2 did not result in a reduction in the prevalence of the activation-related closed time component.

At higher concentrations, BI-2 caused inhibition of channel activity by enhancing apparent desensitization. From a mechanistic standpoint, this effect was similar to that observed for pregnenolone sulfate (Akk et al., 2001) and 3β-hydroxysteroids (Wang et al., 2002). Thus, benz[*e*]indene BI-2 has dual potentiating and inhibitory effects on the $GABA_A$ channel.

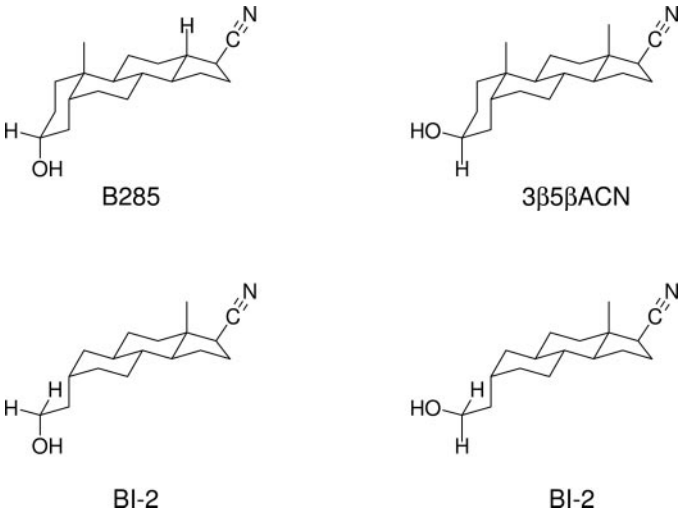

Fig. 1. Structures of B285, 3β5βACN, and BI-2. B285 is a 5β-reduced, 3α-hydroxysteroid that potentiates GABA action (Akk et al., 2004). 3β5βACN is a 5β-reduced, 3β-hydroxysteroid that inhibits GABA action (Wang et al., 2002). BI-2 is a flexible compound that can mimic either a potentiating 5β-reduced, 3α-hydroxysteroid when in the conformation shown at the bottom left or an inhibiting 5β-reduced, 3β-hydroxysteroid when shown in the conformation at the bottom right.

Materials and Methods

Molecular Biology and Drugs. The experiments were carried out on $GABA_A$ receptors consisting of rat α1, β2, and γ2L subunits that were subcloned into a cytomegalovirus promoter-based expression vector pcDNAIII (Invitrogen, Carlsbad, CA) and transiently expressed in human embryonic kidney (HEK) 293 cells. Subunits containing mutations (α1Q242W, α1N408A/Y411F, and α1V256S) were generated using the QuikChange kit (Stratagene, La Jolla, CA). To verify the mutations, the constructs were sequenced over the entire coding region.

The transfection procedures have been described in detail previously (Akk, 2002). In brief, a total of 7.5 μg of cDNA in the ratio of 1:1:1 or 1:1:10 (α:β:γ) per 35-mm dish was used for a calcium phosphate precipitation-based transfection. In some experiments, the cells were transfected with α and β subunits in the ratio of 1:1 (total of 5 μg of cDNA per dish). The cells were incubated with the precipitate at 37°C in 5% CO_2 for ~20 h, after which the medium was replaced. The experiments were carried out at 24 to 72 h after the start of transfection. Most drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). BI-2 was synthesized as described previously (Han et al., 1995).

Electrophysiology. The electrophysiological experiments were carried out using standard cell-attached single-channel patch-clamp and whole-cell voltage-clamp techniques. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. The pipette solution contained 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, and 10 mM HEPES, pH 7.4. In whole-cell recordings, the recording pipette was filled with an internal solution containing 140 mM CsCl, 4 mM NaCl, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES, pH 7.4.

In single-channel recordings, the agonist (GABA) and modulator (BI-2 and neuroactive steroids) were present in the pipette medium. In whole-cell recordings, the drugs were applied using an SF-77B fast-perfusion stepper system (Warner Instruments, Hamden, CT).

Pipettes were pulled from borosilicate glass capillaries (WPI, Sarasota, FL), coated with Sylgard (Dow Corning, Midland, MI) and fire-polished immediately before recordings. The pipettes typically had a tip resistance of 3 to 5 MΩ. In whole-cell recordings, no compensation was made to either series resistance or pipette capacitance. Most recordings were made from cells with maximal currents <2.5 nA, and there was no indication of correlation between the maximal response and the concentration of GABA producing half-maximal response, suggesting that series resistance errors did not significantly affect the results.

In single-channel recordings, the pipette potential was held at +60 to +80 mV, for a total voltage across the patch membrane of approximately −100 mV. In whole-cell recordings, the cells were clamped at −60 to −20 mV. All experiments were carried out at room temperature.

The channel activity was recorded using an Axopatch 200B amplifier. The single-channel records were low-pass filtered at 10 kHz and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software (Molecular Devices, Sunnyvale, CA). The whole-cell current traces were low-pass filtered at 2 kHz and acquired at 10 kHz.

Data Analysis. The single-channel kinetic analysis has been described in detail previously (Akk et al., 2001, 2004). For event detection, the currents were idealized at 2 to 3 kHz. Most analysis was carried out on single-channel clusters (i.e., relatively brief episodes of channel activity with high open probability). The clusters were isolated from the rest of the recording and from each other by applying a critical closed time interval (τ_{crit}). Channel events sepa-

rated from each other by closed intervals longer than τ_{crit} were considered to originate from separate clusters, whereas openings separated with closed intervals shorter than τ_{crit} were considered part of the same cluster. Due to overall high activity levels, in many cases clusters were identified visually as condensed series of openings separated from other such episodes by closed times of >200 ms. In addition, to exclude activity from receptors lacking the γ subunit, we discarded single-channel clusters in which the main conductance level was characteristic of receptors consisting of $\alpha\beta$ subunits (Verdoorn et al., 1990). Samples of visual cluster identification in a record obtained in the presence of 50 μM GABA are given in Fig. 3.

Three major sets of parameters were estimated from cluster analysis. The intracluster open and closed times were estimated using a maximum likelihood method (QuB Suite; www.qub.buffalo.edu), which incorporates a correction for missed events. The open times were fitted by sums of two or three exponentials, whereas for intracluster closed times, a three-component fit was optimal. Cluster durations were estimated as the time interval from the beginning of the first opening to the termination of the last opening within a cluster.

The analysis of whole-cell currents was based on determining the peak current amplitude and the desensitization time constant using pClamp 9.0 software package. The decay was fit to the sum of a single exponential and a constant, over the data from approximately 95% of peak to the end of application.

Data presented in the text are mean ± S.D. The best-fit parameters and standard deviations for the concentration-effect data were obtained using program NFIT (The University of Texas Medical Branch at Galveston, Galveston, TX).

Results

BI-2 Potentiates GABA-Activated Receptors. Initial experiments on the activation of $\alpha1\beta2\gamma2L$ $GABA_A$ receptors were carried out at 1 to 3000 μM GABA. Sample current traces from one cell are shown in Fig. 2A. To construct the GABA concentration-response curve, we first normalized the current amplitudes to the peak current obtained with 1 or 3 mM GABA in the same cell. The normalized current responses were then averaged and fitted with the following equation: response = $1/(1 + (EC_{50}/[GABA])^{n_H})$, where EC_{50} corresponds to the midpoint of the concentration-response curve, [GABA] is the concentration of GABA, and n_H is the Hill coefficient. From the data obtained from 5 to 15 cells, we estimate an EC_{50} of 9.4 ± 1.1 μM and a Hill coefficient of 0.9 ± 0.1 (Fig. 2B).

A previous study showed that the regular transfection approach, which we have used successfully in single-channel recordings and in which the α, β, and γ subunits are used in the ratio of 1:1:1, can result in a significant functional contribution from $\alpha\beta$ receptors (Boileau et al., 2003). Our single-channel studies, in which occasional lower amplitude cur-

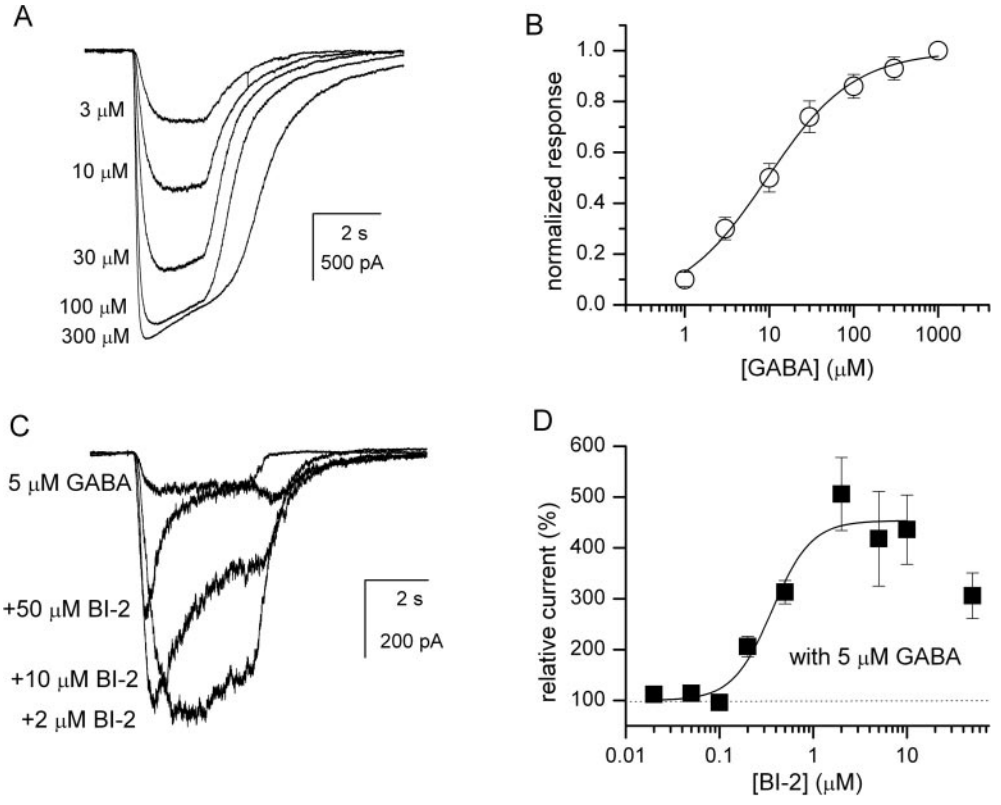

Fig. 2. Benz[e]indene BI-2 potentiates the peak response from $GABA_A$ receptors. A, sample responses from an HEK cell transfected with $\alpha1\beta2\gamma2L$ subunits. GABA was applied for 2 s; successive responses were separated by 30-s intervals of washout. The cell was clamped at −40 mV. B, GABA dose-response curve. The data points represent mean ± S.E.M. for data from 5 to 15 cells per data point. The curve was fitted to the equation in *BI-2 Potentiates GABA-Activated Receptors*. The best-fit parameters are EC_{50} = 9.4 ± 1.1 μM and n_H = 0.9 ± 0.1. C, sample response from a cell exposed to 5 μM GABA in the absence and presence of 2, 10, or 50 μM BI-2. Drug exposures lasted 4 s; successive applications were separated by 30 s washouts, and the cell was clamped at −30 mV. The data demonstrate that micromolar concentrations of BI-2 potentiate the peak response; however, enhanced apparent desensitization is seen in the presence of high concentrations of BI-2. D, BI-2 dose-response curve for potentiation of peak current. The cells were exposed to 5 μM GABA and 0.02 to 50 μM BI-2. The data points represent mean ± S.E.M. for data from 4 to 16 cells per data point. The curve was fitted to the equation under *BI-2 Potentiates GABA-Activated Receptors*; however, the 50 μM data point was excluded from the fit. The best-fit parameters are maximal potentiation = 454 ± 24%, EC_{50} = 0.4 ± 0.1 μM, and n_H = 2.1 ± 0.7. The dotted line corresponds to current levels seen in the absence of BI-2.

rents, likely corresponding to $\alpha\beta$ receptor activity, can be seen, tend to agree with this finding (Fig. 3). Although in single-channel recordings the activity of γ-less receptors can easily be identified and excluded from further analysis, because of its lower conductance, no such post hoc correction exists for whole-cell recordings, where all types of channel activity contribute to the overall current response. Therefore, it has been suggested that the use of a transfection mix with elevated γ-to-α and -β levels may help to reduce the formation of $\alpha\beta$ receptors (Boileau et al., 2003).

To test this, we used two approaches to estimate the contribution of $\alpha\beta$ receptors to the overall macroscopic response from cells transfected with $\alpha\beta\gamma$ subunits. First, we compared the blocking effect of Zn^{2+} on currents from cells transfected with different $\alpha\beta\gamma$ subunit ratios. It is well known that the presence of the γ subunit in the receptor complex results in resistance to the blocking effect of Zn^{2+} (Draguhn et al., 1990); thus, a significant inhibition of peak current would be an indication of the contribution of $\alpha\beta$ receptors to the electrophysiological response. Our data show that 100 μM $ZnCl_2$ was equally ineffective at blocking currents from cells transfected with $\alpha\beta\gamma$ subunits with 1:1:1 ratio (96 $\pm$ 5% of control remaining; n = 6 cells) as from cells transfected with $\alpha\beta\gamma$ subunits with 1:1:10 ratio (92 $\pm$ 9% of control remaining; n = 7 cells). For comparison, Zn^{2+} inhibited the peak current to 52 $\pm$ 9% of control (n = 5 cells) in cells transfected with $\alpha\beta$ subunits.

Second, we examined the ability of BI-2 to potentiate currents elicited by a saturating dose (1 mM) of GABA. This experiment was designed around the idea that the maximal open probability of $\alpha\beta$ receptors, in the presence of GABA, is less than that of $\alpha\beta\gamma$ receptors for which we have estimated the maximal open probability to be >0.8 (Serafini et al., 2000; Steinbach and Akk, 2001). As a result, many potentiators retain their ability to potentiate the $\alpha\beta$ receptor even in the presence of saturating GABA, and a significant potentiation of peak current from cells transfected with $\alpha\beta\gamma$ subunits would indicate a presence of $\alpha\beta$ receptors. Our data demonstrate that 2 μM BI-2 was ineffective at potentiating the peak current elicited by 1 mM GABA from cells transfected with $\alpha\beta\gamma$ subunits. The average peak current in the presence of GABA + BI-2 was 104 $\pm$ 8% (n = 6 cells) of control in cells transfected with 1:1:1 ratio and 101 $\pm$ 11% (n = 7 cells) of control in cells transfected with 1:1:10 ratio for the $\alpha\beta\gamma$ subunits. For comparison, 2 μM BI-2 potentiated the peak current elicited by 1 mM GABA from cells transfected with $\alpha\beta$ subunits to 206 $\pm$ 51% (n = 5 cells) of control.

These findings indicate that, in our hands, the excess of γ relative to α and β subunits does not lead to further incorporation of the γ subunit into receptor complexes. Based on these findings, we have carried out all further experiments using the subunit ratio of 1:1:1 (α:β:γ) in our transfections.

Previous studies have shown that benz[*e*]indenes, at micromolar concentrations, potentiate currents elicited by GABA (Hu et al., 1993; Han et al., 1995). Here, we confirm that the application of BI-2, at low micromolar concentra-

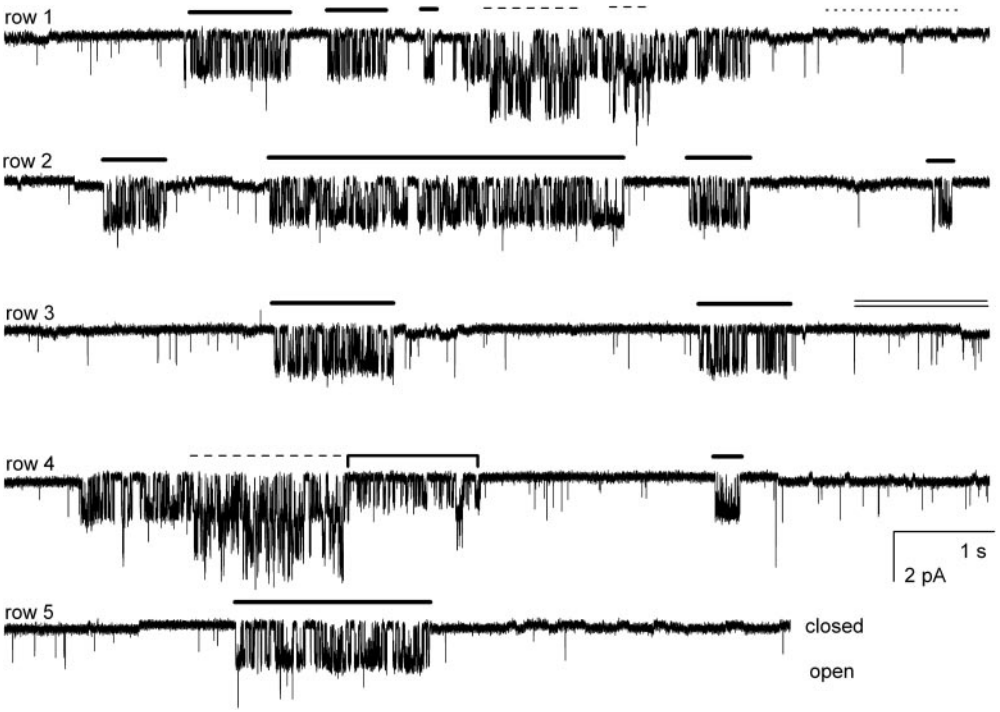

Fig. 3. Heterogeneity in single-channel recordings. The figure shows five consecutive 10-s segments. Channel openings are downward deflections. The currents were recorded in the cell-attached configuration with 50 μM GABA in the pipette. The pipette potential was +60 mV. Single-channel clusters (shown with thick solid lines above the current traces) were identified visually, extracted from the record, and saved for further analysis. The record also contains overlapping currents with two or more channels open simultaneously (dashed lines in rows 1 and 4), and channel openings with intermediate amplitudes (bracket in row 4), possibly representing activity from receptors lacking the γ2 subunit. In addition, background openings with low open probability can be seen (e.g., double line in row 3). The origin of these openings is unknown, but indirect evidence suggests they may represent activity from misassembled $GABA_A$ receptors. Although the background openings occasionally superimpose single-channel clusters used in the analysis, their contribution to the idealized intracluster activity, and thus error in the estimated parameters is negligible. Finally, this record contained channel openings with amplitudes on par with baseline noise (e.g., dotted line in row 1). A typical single-channel recording contains single-channel clusters with any combination of additional types of activity (i.e., overlapping currents, openings with intermediate amplitudes, and background openings).

tions, potentiates currents elicited by GABA. Figure 2C shows sample recordings from a cell exposed to 5 μM GABA in the absence and presence of 0.02 to 50 μM BI-2. The BI-2 potentiation curve had a bell-shaped form with the maximal potentiating effect (506 ± 143%, n = 4 cells, 100% equals to no effect) taking place in the presence of 2 μM BI-2 (Fig. 2D). Fitting the curve (excluding the 50 μM data point) to the equation above yielded a maximal response of 454 ± 24% with a half-maximal effect at 0.4 ± 0.1 μM BI-2 and a Hill coefficient of 2.1 ± 0.7.

BI-2 Potentiates GABA-Activated Receptors through an Effect on Channel Open Times. In cell-attached single-channel recordings, the activation of $\alpha1\beta2\gamma2L$ $GABA_A$ receptors by GABA, at concentrations of 50 μM and greater, results in clearly distinguishable episodes of high-frequency openings and closings (i.e., clusters). The individual clusters can last for several seconds and are separated from other such episodes by periods of inactivity whose durations depend on the number of receptors in the patch (Fig. 3). Each cluster is a product of activation of a single ion channel, although neighboring clusters do not necessarily originate from the same channel. The ability to recognize currents arising from the activation of a single channel allows the investigator to study changes in channel open and closed times in response to the application of a modulator and negates the contribution of an increase in the number of active channels in the patch (e.g., because of changes in desensitization) to changes in channel closed times. Thus, studying an effect of a drug on single-channel clusters can yield more precise mechanistic insight into the nature of the effect.

To evaluate the mechanism of potentiation for BI-2, we carried out single-channel patch-clamp recordings in the presence of 50 μM GABA and 0.1 to 50 μM BI-2. The major result is that an increase in the concentration of BI-2 leads to an increase in the channel mean open duration (Fig. 4). In the presence of 0.1 μM BI-2 (and 50 μM GABA), the mean open duration is 3.2 ± 0.7 ms (n = 4 patches). This value is similar to the control data obtained with 50 μM GABA (3.3 ± 1.7 ms; Akk et al., 2005). As the concentration of BI-2 is raised, the mean open duration increases, reaching its peak (9.9 ± 3.8 ms; n = 4 patches) at 2 μM.

Previous single-channel studies have described the complexity of intracluster kinetics. The channel open events comprise three classes of openings with mean durations of approximately 0.3 ms (designated as OT_1), 2 ms (OT_2), and 8 ms (OT_3) (Akk et al., 2001; Fisher, 2004). Studies on the mechanisms of action of potentiating neuroactive steroids have shown that potentiation results from a selective increase in the duration and relative contribution of the longest lived open time component, OT_3 (Akk et al., 2004). This results in an increase in the mean open duration, and, consequently, the cluster open probability, leading to enhancement in the peak whole-cell response.

We now describe how BI-2 influences the parameters of each of the open time components. The properties of the shortest lived open time component, OT_1, were not affected by the presence of BI-2 (Fig. 5, A and B). Linear regression analysis of the data obtained in the presence of 0.1 to 50 μM BI-2 gave a nonsignificant correlation between the duration of OT_1 and BI-2 concentration (R^2 = 0.007; p = 0.70) and the fraction of OT_1 and BI-2 concentration (R^2 = 0.008; p = 0.68). The mean duration of OT_1 at 0.1 to 50 μM BI-2 was 0.34 ± 0.14 ms (n = 25 patches), and the mean fraction of OT_1 was 0.31 ± 0.11.

It is noteworthy that the OT_2 and OT_3 components could be reliably separated only at BI-2 concentrations up to 0.5 μM. In nine patches recorded in the presence of 50 μM GABA and 0.1 to 0.5 μM BI-2, the mean duration of OT_2 was 2.2 ± 1.0 ms with an average fraction of 0.31 ± 0.12. Under the same conditions, the mean duration of OT_3 was 8.5 ± 3.5 ms, and the fraction of OT_3 was 0.36 ± 0.14.

At higher [BI-2], the open time histograms fitted with three exponentials had, in most cases, log-likelihoods not significantly higher than those obtained in fits with two exponentials. Thus, in Fig. 5, C and D, we show only the results of two exponential open time fits for data obtained at BI-2 concentrations of 1 μM and higher.

In the range of 1 to 50 μM BI-2, the duration of the longer lived open time component (designated as OT_{2-3}) was relatively uniform. There was a tendency toward higher durations at intermediate concentrations, with an apparent maximum at 2 μM BI-2 (mean duration, 13.2 ± 3.9 ms; four patches), but this was not statistically different from the OT_{2-3} duration at 5, 10, or 50 μM BI-2. It should be noted, however, that the duration of OT_{2-3} at 2 μM BI-2 was different from both OT_2 (p = 0.002) and OT_3 (p = 0.03) observed in the presence of 0.1 μM BI-2.

The fraction of openings in the shortest component of open times (OT_1) did not change with [BI-2] (see above), and, as

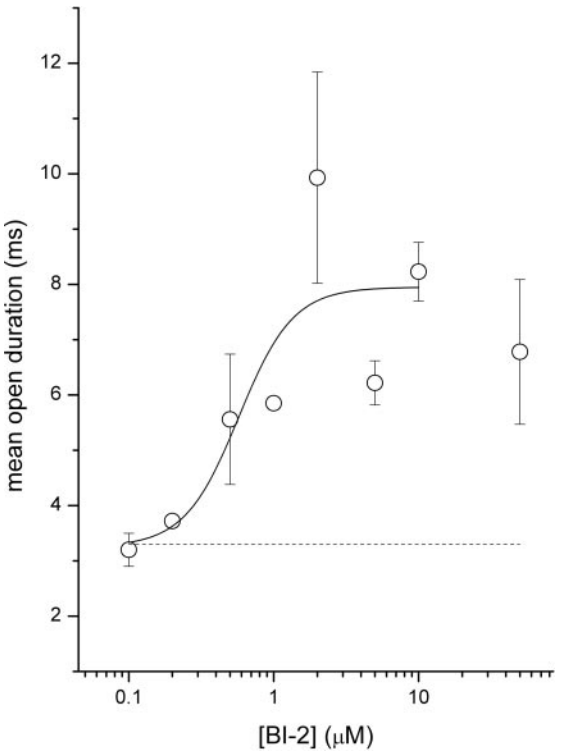

Fig. 4. Mean open duration depends on the concentration of BI-2. The receptors were exposed to 50 μM GABA and 0.1 to 50 μM BI-2. The data points represent means ± S.E.M. The curve was fitted to the equation under *BI-2 Potentiates GABA-Activated Receptors* with an offset; however, the 50 μM data point was excluded from the fit. The best-fit parameters are EC_{50} = 0.57 ± 0.43 μM, n_H = 2.3 ± 3.9, OT_{min} = 3.3 ± 1.9 ms, and OT_{max} = 8.0 ± 2.5 ms. The dashed line corresponds to the mean open duration in the absence of BI-2.

expected, the fraction in OT_2 or OT_3 (for [BI-2] < 1 μM) or in $OT_{2\text{-}3}$ was constant.

In sum, our single-channel data demonstrate that BI-2 acts by increasing the channel mean open duration. Two major effects underlie the increase. First, BI-2 acts by eliminating one class of openings (OT_2). Several kinetic mechanisms can result in such phenomenon. Our data (Fig. 5D) favor a mechanism in which BI-2 enhances the prevalence of OT_3 offset by a decrease in the fraction of OT_2. As a result, at high (1 μM and above) BI-2 concentrations, the OT_2 component becomes vanishingly small and no longer identifiable, leading to a single, long-duration open time component. It should be noted here that many neuroactive steroids act by increasing the fraction of OT_3 with a simultaneous decrease in the fraction of OT_2, although the loss of OT_2-type openings was never complete in those studies (Akk et al., 2004, 2005). The second action of BI-2 was to increase the duration of the longer lived open time component, $OT_{2\text{-}3}$, over the durations of either OT_2 or OT_3.

In the presence of just GABA, the mean open duration of the $GABA_A$ channel is similar to OT_2 because this class of openings is a prominent component in the open time histograms, and because the other two components—OT_1 (brief) and OT_3 (long)—have a tendency to cancel each others influence on the mean open duration. In the presence of GABA + BI-2, however, the difference between OT_2 and OT_3 is eliminated, resulting in a single, dominant class of long openings ($OT_{2\text{-}3}$), and the mean open duration approaches the mean duration of that component.

BI-2 Has Only Minor Effects on Intracluster Closed Times. The observation that the mean open duration is increased requires that BI-2 must decrease the overall rate for the open channel to enter nonconducting states. Indeed, the rate for terminating an open event decreases from approximately 400 s^{-1} in the presence of 0.1 μM BI-2 to approximately 160 s^{-1} with 10 μM. To gain more insight, we then examined the actions of BI-2 on the distributions of closed time components within clusters.

Previous kinetic analysis has identified two main classes of intracluster closed times. The first class contains the types of channel closures that are associated with agonist association and dissociation (designated as CT_β). The mean duration of this type of closed times depends, among other parameters, on agonist concentration. In the presence of higher doses of agonist, agonist binding proceeds more rapidly, and the corresponding closed dwells are briefer. The second type of channel closures is not part of the activation pathway but rather takes place after channel opening (designated as CT_1 and CT_2). Little is known of the origin of such states, but possible mechanisms include block, short-lived desensitized and other types of nonconducting states (Twyman et al., 1990; Haas and Macdonald, 1999; Steinbach and Akk, 2001). The durations of these closed states are briefer than the duration of CT_β at most GABA concentrations, and they do not depend on agonist concentration.

The durations of two shorter lived closed time components (CT_1 and CT_2) are mainly unaffected by 0.1 to 10 μM BI-2 (Fig. 6, A–D). Analysis by linear regression suggests no correlation between the duration ($R^2 = 0.05$; $p = 0.98$) or fraction of CT_1 ($R^2 = 0.002$; $p = 0.85$) and the concentration of BI-2. The mean duration of CT_1 was 0.16 ± 0.05 ms at 0.1 to 10 μM BI-2 ($n = 20$ patches). The mean fraction of CT_1 was 0.54 ± 0.12. Likewise, there was no indication of correlation between the duration ($R^2 = 0.19$; $p = 0.06$) or fraction of CT_2 ($R^2 = 0.04$; $p = 0.42$) and the concentration of BI-2. The mean durations and fractions of CT_2 across BI-2 concentrations were 1.6 ± 0.4 ms and 0.27 ± 0.06, respectively.

The duration and fraction of CT_β also showed relatively little dependence on the concentration of BI-2 (Fig. 6, E–F). Although there was a slight trend toward longer CT_β durations at higher BI-2 concentrations, this relationship had a slope that was not significantly different from zero (R^2 =

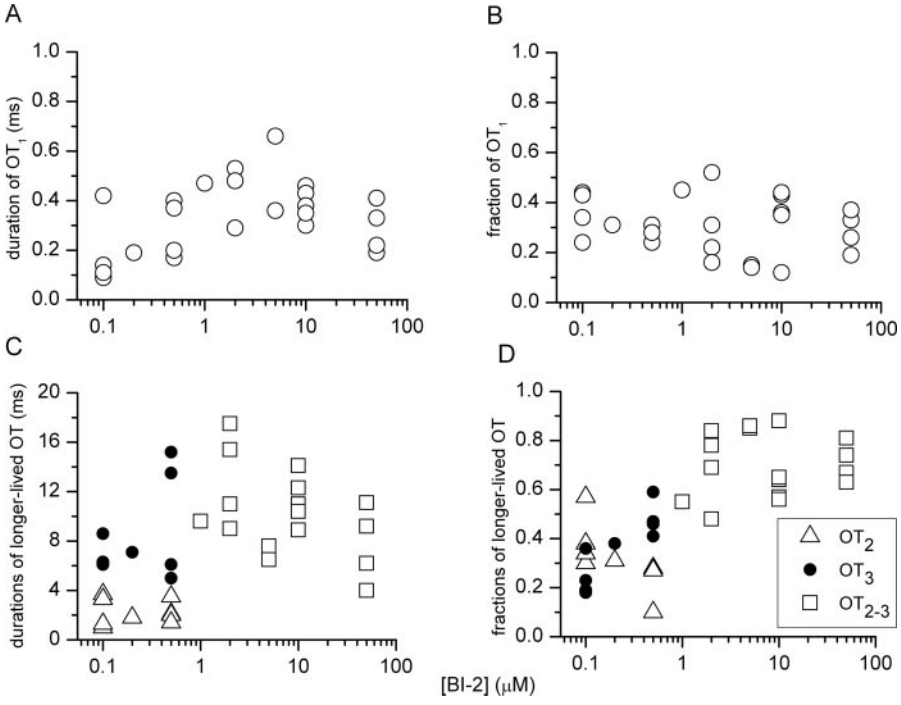

Fig. 5. BI-2 enhances the duration and prevalence of the longer lived open time component. The receptors were exposed to 50 μM GABA and 0.1 to 50 μM BI-2. A, mean durations of the shortest lived open time component (OT_1). Each symbol represents data from a single patch. The mean OT_1 duration across modulator concentrations was 0.34 ± 0.14 ms. No concentration-dependent effect in the duration of OT_1 was noted (also see text). B, mean fractions of the OT_1 component. The mean value across BI-2 concentrations was 0.31 ± 0.11. No concentration-dependent effect on the fraction of OT_1 was observed (also see text). C, mean durations of OT_2 and OT_3 were estimated from fitting the open time histograms to sums of three exponentials at BI-2 concentrations of 0.1 to 0.5 μM. At higher concentrations (1–50 μM), the histograms were fitted to the sums of two exponentials. The longer duration component from such fits is shown as $OT_{2\text{-}3}$. At its peak (2 μM BI-2), the mean duration of $OT_{2\text{-}3}$ was greater than that of OT_2 or OT_3. D, mean fractions of OT_2, OT_3, and $OT_{2\text{-}3}$ were estimated from fits to sums of three (0.1–0.5 μM) or two (1–50 μM) exponentials. The data suggest that the fraction of OT_2 decreases, whereas the fraction of OT_3 increases with elevated concentrations of BI-2. The data indicate that at [BI-2] > 0.5 μM, the fraction of OT_2 becomes negligible resulting in a single long-lived component, $OT_{2\text{-}3}$.

0.19, $p = 0.06$). The lack of effect on the duration of CT_β by benz[e]indene BI-2 agrees with our previous findings on neuroactive steroids (Akk et al., 2004, 2005).

It is interesting that the data also suggest that BI-2 has little effect on the fraction of CT_β (Fig. 6F). The averaged value for the fraction of CT_β at 0.1 to 5 μM BI-2 was 0.22 ± 0.10. For comparison, in the absence of modulators, the fraction of CT_β ranges from 0.17 to 0.37 (Steinbach and Akk, 2001). There was a reduction in the fraction of CT_β at 10 μM BI-2 (0.08 ± 0.03), but this value should be taken with some caution because the mechanism of inhibition by BI-2 (shorter clusters; see below) results in a bias against long-interval durations, which are selectively lost as the cluster durations decrease. Even so, linear regression analysis on the whole data set (0.1–10 μM BI-2) gave a nonsignificant correlation between the prevalence of CT_β and BI-2 concentration ($R^2 = 0.17$; $p = 0.08$).

This finding is in striking contrast with our previous studies on neuroactive steroids in the presence of which the prevalence of the CT_β component was reduced in parallel with the effects on the channel open durations (Akk et al., 2004, 2005). The reduction in contribution of the CT_β component results from a selective decrease in the channel closing rate in the presence of steroid, from approximately 100 s^{-1} to approximately 10 to 20 s^{-1} (Akk et al., 2004). In contrast, the channel closing rate in the presence of 2 to 5 μM BI-2 was 36 ± 25 s^{-1} with no significant change in its prevalence. The selective reduction in the fraction of the relatively long-lived CT_β constitutes an additional factor contributing to the overall potentiating effect of steroids. It seems from our present findings that benz[e]indene BI-2 potentiates the $GABA_A$ receptor function exclusively through the open duration effect.

What Causes the Inability of BI-2 to Affect Closed Times? The inability of BI-2 to affect the rate of occurrence of CT_β may be caused by the inability of BI-2 to interact with a binding site ("binding mechanism") or by the inability of such interactions to result in changes in closed times ("transduction mechanism"). To help to distinguish between the two mechanisms, we examined channel modulation by BI-2 in the presence of B285 (Fig. 1), a neuroactive steroid that has been shown to affect the prevalence of CT_β (Akk et al., 2004). In these experiments, we coapplied 500 nM B285 and 5 μM BI-2 along with 50 μM GABA. The concentration of B285 was selected to cause an almost full suppression of the CT_β component (Akk et al., 2004), whereas the concentration of BI-2 was selected to cause a near-maximal effect in whole-cell potentiation (Fig. 2D) and on channel open durations as determined from single-channel patch clamp (Fig. 4). We reasoned that an increase in the prevalence of CT_β could be interpreted as BI-2 competitively inhibiting the ability of B285 to reduce the fraction of CT_β. On the other hand, the absence of effect on CT_β would be an indication of the inability of BI-2 to interact with the site through which the closed time effect is mediated.

The results of the analysis of single-channel data from four patches agree with the latter hypothesis. In the presence of 500 nM B285 + 5 μM BI-2, the rate of entry into the longest closed state (closing rate) was 13.2 ± 4.3 s^{-1}, and the CT_β component constituted 11 ± 3% of all intracluster closed times. These values are in line with previously published data (22.2 s^{-1}; 12%; Akk et al., 2004) and more recent control data (18.5 s^{-1}; 11%; Fig. 7) for 50 μM GABA + 500 nM B285, showing that 5 μM BI-2 was unable to block the closed time effect exerted by a neuroactive steroid. Thus, the data suggest that benz[e]indene compound BI-2 has a very low affinity for the site mediating the closed time effect rather than binding to the site with high affinity but being incapable of causing the conformational change underlying the closed time effect.

Does BI-2 Potentiate Receptor Function by Acting through the Steroid Binding Site? Although several studies have indicated the importance of the membrane-spanning

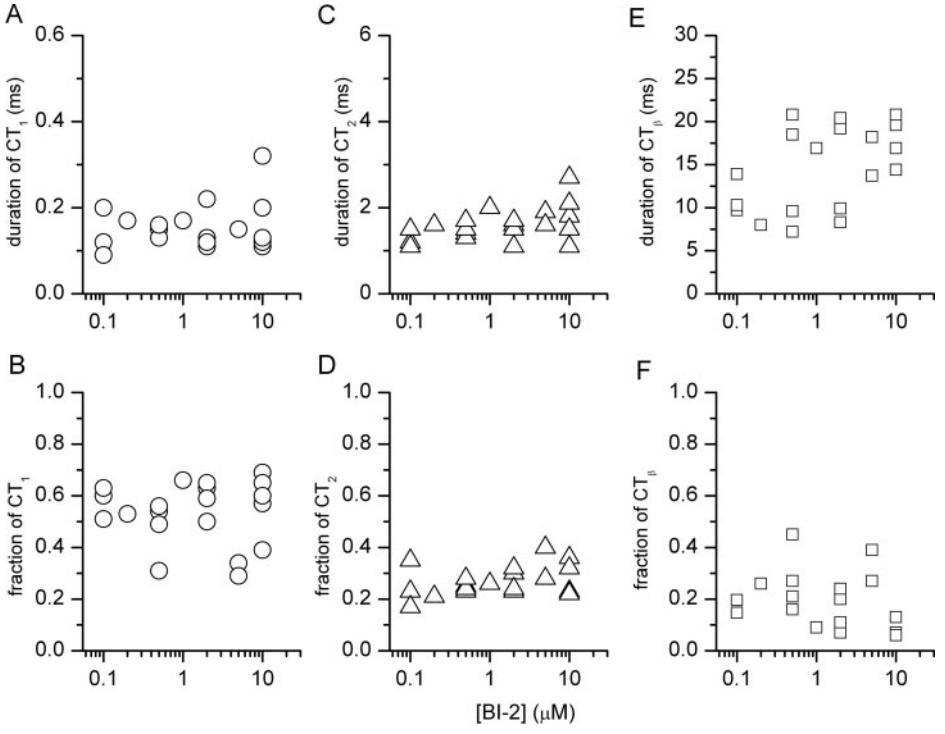

Fig. 6. BI-2 does not affect the properties of the intracluster closed time components. The receptors were exposed to 50 μM GABA and 0.1 to 10 μM BI-2. A, mean durations of the shortest lived closed time component (CT_1). Each symbol represents data from a single patch. The mean CT_1 duration across modulator concentrations was 0.16 ± 0.05 ms. B, mean fractions of the CT_1 component. The mean value across BI-2 concentrations was 0.54 ± 0.12. C, mean durations of CT_2. The mean value across BI-2 concentrations was 1.6 ± 0.4 ms. D, mean fractions of CT_2. The mean value across BI-2 concentrations was 0.27 ± 0.06. E, mean durations of CT_β. The mean value across BI-2 concentrations was 14.5 ± 4.9 ms. F, mean fractions of CT_β. The mean value across BI-2 concentrations was 0.19 ± 0.11. No BI-2 concentration-dependent effect on any of the parameters was observed (also see text).

domains of the GABA-activated receptor in the actions of neuroactive steroids (Rick et al., 1998; Morris and Amin, 2004; Akk et al., 2005), it was only very recently that Hosie et al. (2005) reported the specific residues in the M1 (Q242) and M4 transmembrane domains (N408 and Y411) of the α subunit that form (at least part of) the binding site for endogenous steroids such as allopregnanolone and tetrahydrodeoxycorticosterone. We have presently used this finding to further examine the commonality among the binding sites for potentiating neurosteroids and benz[*e*]indenes.

To do so, we compared the ability of BI-2 to potentiate currents from wild-type and mutant receptors. The experiments were carried out at GABA concentrations corresponding to $\sim EC_{25}$ (Fig. 2B for wild type; Fig. 8A for mutant receptors) and 2 μM BI-2. The results demonstrate that the mutations have a dramatic effect on channel potentiation. For the wild-type receptor, the response in the presence of BI-2 was approximately 5 times the control, whereas for the α1Q242W mutant the response was only 0.9 $\pm$ 0.1 of control, and for α1N408A/Y411F it was 1.3 $\pm$ 0.1 times control. Together, the results suggest that neurosteroids and benz[*e*]indene BI-2 potentiate the $GABA_A$ receptor by interacting with the same set of binding sites.

As a control, we also compared channel potentiation by allopregnanolone and a 5β-reduced neuroactive steroid, B285, in wild-type and mutant receptors. In agreement with previous data (Hosie et al., 2005), the mutations abolished potentiation by allopregnanolone (Fig. 8B). However, for B285, only the α1Q242W mutation eliminated potentiation, whereas the steroid potentiated the α1N408A/Y411F double mutant on par with the wild-type receptor.

High Concentrations of BI-2 Inhibit GABA-Activated Receptors. The whole-cell recordings demonstrated that receptor exposure to high concentrations of BI-2 (10–50 μM) resulted in an increase in the apparent rate of desensitization (Fig. 2C). Receptors bathed with 50 μM GABA + 50 μM BI-2 had an apparent desensitization time constant (τ_D) of 438 $\pm$ 150 ms (n = 5 cells). For comparison, in the absence of BI-2, the desensitization decay had a time constant of 5163 $\pm$ 2276 ms (n = 7 cells). Such a phenomenon, a more rapid

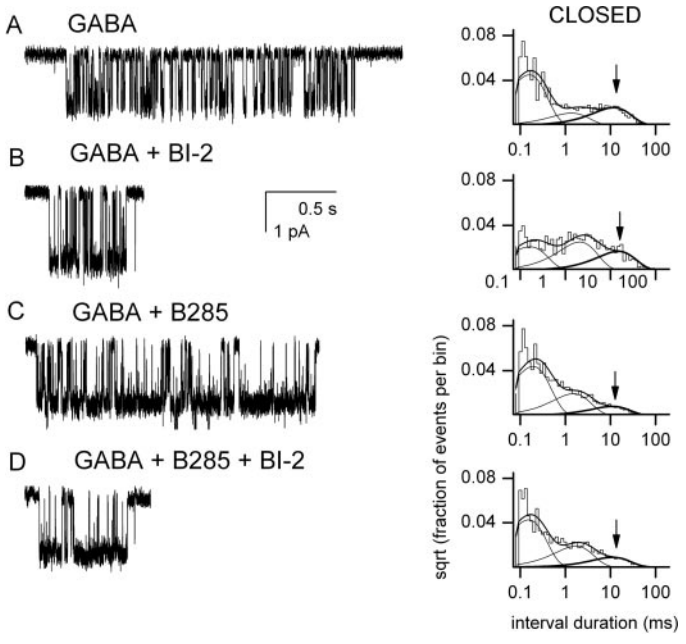

Fig. 7. Exposure to BI-2 does not interfere with the ability of B285 to produce a decrease in the prevalence of the longest closed time component. Sample clusters and the corresponding closed time histograms are shown for receptors exposed to GABA, GABA + BI-2, GABA + B285, and GABA + B285 + BI-2. Channel openings are shown downward. The drug concentrations were 50 μM GABA, 5 μM BI-2, and 0.5 μM B285. The closed time histograms were fitted to sums of three exponentials. The longest closed time component (CT_β) is indicated with an arrow. The presence of BI-2 does not reduce the fraction of CT_β (compare B and A) and does not interfere with the ability of a 5β-reduced steroid (compare D and C) to reduce the fraction of CT_β. In A, the time constants are 0.15 ms (63%), 1.3 ms (15%), and 10.9 ms (22%). In B, the time constants were 0.15 ms (34%), 1.9 ms (40%), and 13.7 ms (27%). In C, the time constants were 0.18 ms (61%), 1.4 ms (27%), and 10.1 ms (11%). In D, the time constants were 0.14 ms (61%), 1.6 ms (27%), and 10.4 ms (12%).

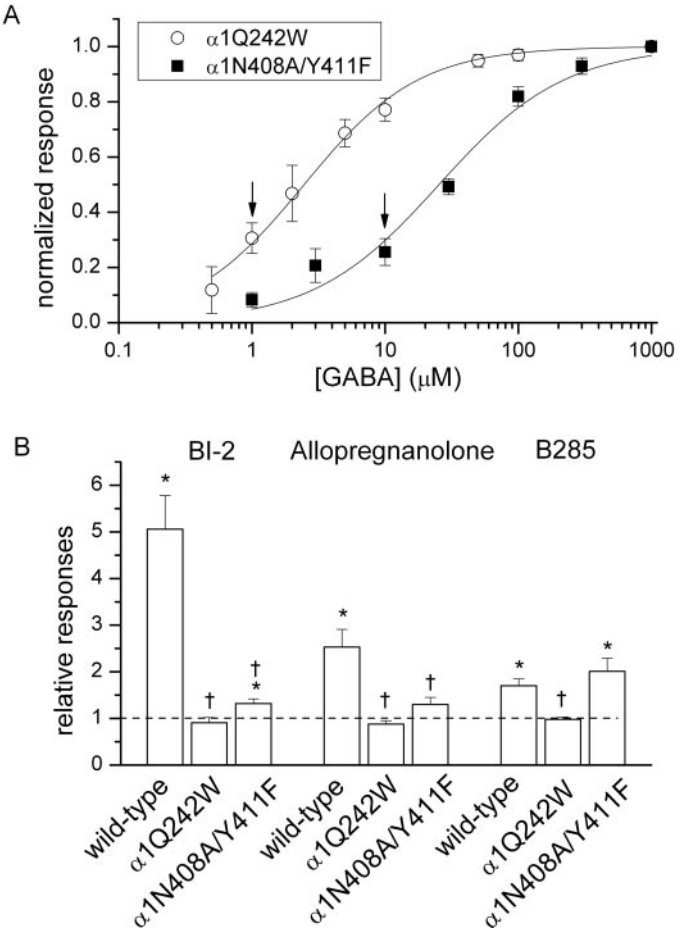

Fig. 8. Mutations to the α subunit, α1Q242W, and α1N408A/Y411F abolish the ability of BI-2 to potentiate $GABA_A$ receptor currents. A, whole-cell dose-response curves for the α1Q242W and α1N408A/Y411F mutant receptors. The data points represent mean $\pm$ S.E.M. for data from 11 (α1Q242W) or nine cells (α1N408A/Y411F) per data point. The curves were fitted to the equation under *BI-2 Potentiates GABA-Activated Receptors*. The best-fit parameters are EC_{50} = 2.5 $\pm$ 0.2 μM and n_H = 1.0 $\pm$ 0.1 for the α1Q242W receptor and EC_{50} = 25.3 $\pm$ 3.9 μM and n_H = 0.9 $\pm$ 0.2 for the α1N408A/Y411F receptor. The arrows indicate the concentrations of GABA at which the effects of modulators were studied. B, relative responses from cells expressing wild-type, α1Q242W, or α1N408A/Y411F mutant receptors. The cells were exposed to 5 μM (wild type), 1 μM (α1Q242W), or 10 μM GABA (α1N408A/Y411F), and 2 μM BI-2, 1 μM allopregnanolone, or 1 μM B285. The columns represent mean $\pm$ S.E.M. for data from 4 to 11 cells. The dashed line corresponds to control current levels seen in the absence of modulators. Asterisks mark statistically significant ($p < 0.05$) differences to a relative response of one (with no modulator present), whereas daggers (note all but B285 on α1N408A/Y411F) indicate significant differences to effects on wild-type receptors, using Student's t test.

apparent desensitization, would have an inhibitory effect on GABA-evoked currents.

The inhibitory effect of BI-2 on whole-cell responses is similar to what has been described previously for pregnenolone sulfate and 3β-hydroxysteroids (Shen et al., 2000; Wang et al., 2002). However, previous studies with pregnenolone sulfate showed that block develops with a rate that is independent of the concentration of GABA (Eisenman et al., 2003). In contrast, the apparent rate of entry into the desensitized state for BI-2 was strongly dependent on the concentration of GABA. The desensitization decay in the presence of 2 μM GABA + 50 μM BI-2 was slower, with a time constant of 3408 $\pm$ 2061 ms (n = 3 cells). In the presence of 10 μM GABA + 50 μM BI-2, the desensitization time constant was 849 $\pm$ 272 ms (n = 5 cells), and at 20 μM GABA + 50 μM BI-2, the desensitization time constant was 518 $\pm$ 140 ms (n = 3 cells). Thus, the ability of BI-2 to inhibit the currents depended on the activation level of the receptor, roughly following the GABA concentration-response curve (Fig. 2B).

Previous studies have shown that a single amino acid mutation in the α1 subunit (V256S) renders the receptor largely insensitive to the inhibitory effect of pregnenolone sulfate and 3β-hydroxysteroids (Akk et al., 2001; Wang et al., 2002). The apparent mechanism of inhibition of these steroids is similar to that observed for BI-2 in that the presence of the modulator increases the apparent rate of desensitization. We therefore examined whether the α1V256S mutation also affects the inhibitory effect of BI-2. Indeed, our data show that the mutation reduces the ability of BI-2 to enhance the rate of desensitization. For the mutant receptor, the τ_D was 12.7 $\pm$ 5.2 s in the presence of 50 μM GABA and 5.4 $\pm$ 1.1 s in the presence of 50 μM GABA + 50 μM BI-2 (n = 4 cells). Although the BI-2-mediated reduction in the duration of τ_D is statistically significant ($p < 0.05$), the approximately 2-fold effect of BI-2 on the mutant receptor currents is significantly different ($p < 0.05$) from the 12-fold effect that BI-2 has on the desensitization time constant in the wild-type receptor.

As described above, the value of τ_D depends on the concentration of agonist. As the concentration of GABA is raised, the apparent rate of desensitization in the presence of BI-2 increases. Therefore, for proper comparison of τ_D estimates in wild-type and mutant receptors, the experiments would have to be carried out at GABA concentrations eliciting similar fractional responses from the two receptor types. A previous study demonstrated that the GABA dose-response curve for the α1V256S receptor is shifted some 20-fold toward lower agonist concentrations compared with the wild-type receptor (Wang et al., 2002). Considering this, we also attempted to estimate the apparent desensitization time constant for the α1V256S receptor in the presence of 2 μM GABA + 50 μM BI-2. Under these conditions, in most cells, little desensitization was observed, and only an approximate value of 30 s for the average desensitization time constant could be obtained. A sample recording, and a comparison to wild-type receptors, is shown in Fig. 9, A and B.

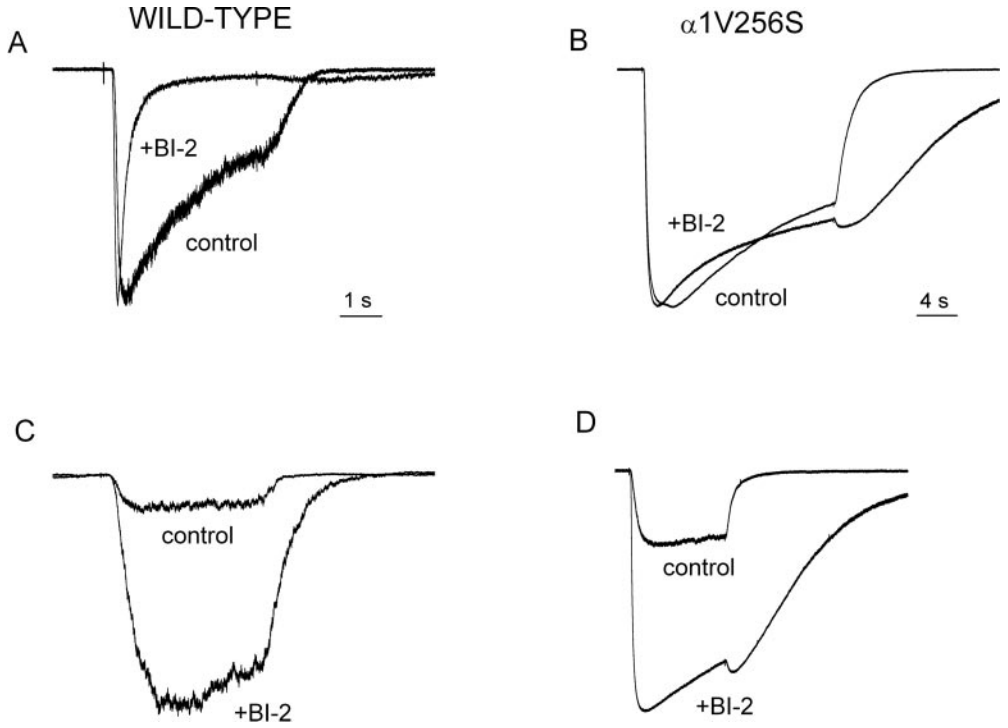

Fig. 9. The α1V256S mutation reduces the inhibitory but not the potentiating effect of BI-2. A, current traces from a cell expressing wild-type receptors in response to 50 μM GABA in the absence and presence of 50 μM BI-2. The presence of BI-2 resulted in an enhanced apparent desensitization. Current decay time courses were fitted to single exponentials with the time constants of 2167 ms (control) and 252 ms (+BI-2). B, current traces from a cell expressing α1V256S mutant receptors in response to 2 μM GABA in the absence and presence of 50 μM BI-2. Current decay time courses were fitted to single exponentials with the time constants of 15.8 s (control) and 8.5 s (+BI-2). C, current traces from a cell expressing wild-type receptors in response to 5 μM GABA in the absence and presence of 2 μM BI-2. The presence of BI-2 resulted in an enhanced peak amplitude (6.2-fold potentiation in this cell). D, current traces from a cell expressing α1V256S mutant receptors in response to 0.2 μM GABA in the absence and presence of 2 μM BI-2. The presence of BI-2 resulted in an enhanced peak amplitude (3.2-fold potentiation in this cell). Different GABA concentrations for wild-type versus mutant receptors were used to account for differences in the dose-response properties. In A and B, the current responses have been normalized to mask the potentiating effect of BI-2. Note the differences in time scales for traces for wild-type and mutant receptors.

The apparent rate of desensitization can be imagined to consist of two components: baseline densensitization (with a rate of k_{+D}) and entry into the blocked or inhibited state (with a rate of [BI-2]k_{+B}). In the absence of BI-2, [BI-2]k_{+B} is zero, and k_{+D} describes the decrease in current response. In contrast, in the presence of high concentrations (e.g., 50 μM) of BI-2, the rate of entry into the blocked state becomes the dominating factor, whereas the contribution from k_{+D} may be considered negligible. The k_{+B} can then be roughly calculated as $(1/\tau_D)/50$, where τ_D is the apparent desensitization time constant, and 50 corresponds to the concentration of BI-2 (in micromolar) used in these experiments. Such calculations give a k_{+B} of 0.05 $\mu M^{-1}\ s^{-1}$ for the wild-type receptor and $<0.0007\ \mu M^{-1}\ s^{-1}$ for the α1V256S mutant receptor. We point out that such kinetic characterization of the effect of BI-2 does not imply that BI-2 blocks the receptor by increasing the rate of desensitization. Our data cannot be used to distinguish between this possibility and a mechanism in which BI-2 inhibits receptor function by introducing a novel, long-lived blocked state.

Overall, the data conclusively demonstrate that the inhibitory effect of BI-2 is dampened by the α1V256S mutation. We have previously concluded that the α1V256 site forms part of the transduction element for inhibition by pregnenolone sulfate (Akk et al., 2001). Thus, the present findings indicate that inhibition by benz[*e*]indene BI-2 shares at least some common elements with inhibition by inhibitory neurosteroids.

The α1V256S mutation had little effect on the potentiating effect of BI-2 (Fig. 9, C and D). In the presence of 0.2 μM GABA, 2 μM BI-2 potentiated peak responses from the mutant receptor to 386 $\pm$ 44% (n = 5 cells).

Finally, we draw attention to the presence of rebound currents during the initial BI-2 washout phase (Fig. 9). The currents are more prominent in the α1V256S than the wild-type receptor, and they resemble those seen in receptors modulated or directly activated by high concentrations of pentobarbital (Akaike et al., 1987). No further characterization of the rebound currents was carried out.

Single-Channel Experiments at High BI-2 Concentrations. Although the major portion of the studies on the inhibitory effect was carried out on whole-cell currents, we also examined the effect of BI-2 on single-channel cluster durations in the wild-type and mutant receptors. The durations of single-channel clusters are considered to be equivalent to the macroscopic desensitization time constant (Sakmann et al., 1980) and can thus provide an additional estimate of the inhibitory actions of BI-2.

Indeed, visual examination of the data (Fig. 10) indicated that the presence of BI-2 resulted in significantly shorter single-channel clusters. Detailed analysis of cluster durations was carried out on data recorded in the presence of 50 μM GABA + 10 μM BI-2. The cluster duration, averaged among four patches, was 202 $\pm$ 55 ms (total of 268 clusters), giving a k_{+B} of 0.50 $\mu M^{-1}\ s^{-1}$. We think this value better characterizes the blocking reaction by BI-2. The reason for the difference between k_{+B} values obtained from the whole-cell and single-channel experiments is not known, but it might reflect differences in the techniques, such as the relatively slow solution exchange in the drug perfusion experiments.

Discussion

The work described in the present article was aimed at elucidating the mechanisms of action of benz[*e*]indene compound BI-2. The experiments, carried out using whole-cell and single-channel recordings, demonstrate that BI-2 has a dual effect on $GABA_A$ receptor function. At low concentrations, the drug acts by potentiating receptor function. At higher concentrations, the effect is inhibition. The effects are sufficiently well separated in terms of drug concentrations, allowing a sharp cut-off between a dose causing receptor potentiation and a dose leading to inhibition.

The inhibitory effect of BI-2 was accomplished via an increase in the apparent rate of desensitization. Whether the presence of BI-2 enhances the "natural" process of desensitization or introduces a novel, long-lived blocked state is unknown. But such a mechanism is, in terms of kinetics, similar to that of pregnenolone sulfate, which has also been shown to enhance apparent desensitization (Shen et al., 2000; Akk et al., 2001). The similarity further extends to the finding that the α1V256S mutation strongly dampens the inhibitory effect of both pregnenolone sulfate and BI-2. However, unlike pregnenolone sulfate, benz[*e*]indene BI-2 affected the $GABA_A$ receptor currents in a manner dependent on the concentration of GABA.

Although desensitization has been shown to shape synaptic currents (Jones and Westbrook, 1995), the mechanism of inhibition described here would be most effective during prolonged activation, such as during repetitive stimulation, or when certain anticonvulsant or anxiolytic drugs are used that enhance GABAergic transmission by prolonging current decay.

The effects of inhibition and potentiation were different in terms of kinetic mechanisms that caused them. Potentiation was the result of an increase in the single-channel cluster open probability that, itself, was caused by an increase in the channel mean open duration. At its peak effect, BI-2 led to a more than 3-fold increase in the mean open duration. The

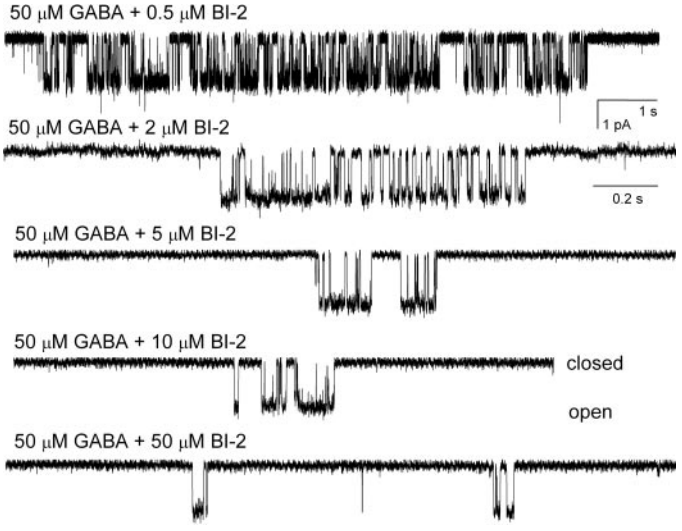

Fig. 10. Exposure to BI-2 results in shorter single-channel clusters. The traces show single-channel currents recorded in the presence of 50 μM GABA and 0.5, 2, 5, 10, or 50 μM BI-2. An increase in the concentration of BI-2 leads to shorter clusters, in addition to the prolongation of the mean open duration. Note that the calibration bar in the top trace differs from the time scale used in the bottom four traces.

effect resembles that of many neuroactive steroids, which also cause prolongation of open times. Similarly to neuroactive steroids (Akk et al., 2004, 2005), BI-2 enhanced the prevalence and duration of the longest open time component. However, in contrast to the actions of neuroactive steroids, which selectively reduce the rate of entry into the closed state associated with activation (that is, channel closing), BI-2, a tricyclic analog of steroids, caused no noticeable change on this parameter.

Based on steroid effects and mutual interactions between the effects of neuroactive steroids, we have previously divided the steroid effects as effects mediated by site A and site B (Akk et al., 2004). Steroid interactions with site A mediate the increase in the fraction of OT_3 and the decrease in the channel-closing rate constant. Steroid interactions with site B mediate the increase in the duration of OT_3. The present data with BI-2 demonstrate that although benz[*e*]indenes increase the duration (site B effect) and fraction (site A effect) of the longest lived open time component, there seems to be no effect on closing rate in the presence of BI-2. Moreover, BI-2 was unable to block the effect of a neuroactive steroid, B285, on the channel-closing rate. These findings strongly suggest that the nominal "site A" effects actually must be split into effects mediated by interactions with two distinct sites. This brings the total number of interaction sites for neuroactive steroids to four: one site for inhibition and three sites for potentiation.

Does BI-2 mediate the effect on channel open times by interacting with the same sites as potentiating steroids do? Several lines of evidence agree with this notion. First, the involvement of modulatory sites for benzodiazepines and picrotoxin has been ruled out by the use of selective antagonists against these sites, which had no effect on potentiation by benz[*e*]indenes (Rodgers-Neame et al., 1992). Second, the potentiating effect of BI-2 and other related compounds is reduced during exposure to (3α,5α)-17-phenylandrost-16-en-3-ol (J. Scaglione, personal communication), a compound that antagonizes the potentiating effect of steroids but not potentiation caused by barbiturates or benzodiazepines (Mennerick et al., 2004). Finally, mutations that have been shown to abolish the potentiating effect of several neurosteroids (Hosie et al., 2005) also reduced potentiation caused by BI-2. However, it should be pointed out that both the steroid antagonist and the mutations may act by affecting a common transduction mechanism, shared by benz[*e*]indenes and neuroactive steroids, whereas the two classes of drugs interact with their own, unique binding sites.

It is worth mentioning here that only one of the mutations (α1Q242W) affected potentiation by a 5β-reduced neuroactive steroid, B285, whereas the double mutant α1N408A/Y411F was ineffective. The exact reason for this difference is unknown, but a partial overlap between the sites for 5α- and 5β-reduced steroids, or dissimilar transduction pathways for different steroids, are possible mechanisms that need to be examined.

The flexibility of BI-2 reasonably explains the dual potentiating and inhibitory actions of this tricyclic compound on $GABA_A$ receptor function. Unlike steroids wherein the 3-hydroxyl group is fixed in either the 3α configuration for neurosteroids with potentiating actions, or in the 3β configuration for neurosteroids with inhibitory actions, the hydroxyl group present in the short flexible chain of BI-2 can mimic steroids with a hydroxyl group in either configuration (Fig. 1). Molecular mechanics calculations (MM2 parameters in Chem3D Ultra 7.0 module of ChemOffice Ultra 2004; CambridgeSoft, Cambridge, MA) show that the conformation of BI-2 that best mimics a 3α-hydroxysteroid is 0.5 kcal/mol more stable that the conformer that best mimics a 3β-hydroxysteroid. This energy difference is insufficient to lock BI-2 into either of these solution conformations or to exclude other low energy conformational states that would be populated by BI-2 at physiological temperatures. Our results indicate that the additional flexibility of the benz[*e*]indene molecule has several consequences for interactions with sites on the $GABA_A$ receptor. BI-2 can interact with two of the potentiating sites to which 3α-hydroxy steroids bind, but not a third site. In addition, BI-2 can bind to the inhibitory site, which recognizes 3β-hydroxysteroids. It is noteworthy that BI-2 is more potent as a potentiator than it is as an inhibitor of $GABA_A$ receptor function. The same is true for steroids in that 3α-hydroxysteroids are more potent at augmenting GABA action than the corresponding 3β-hydroxysteroids are at diminishing GABA action. In the future, it will be interesting to determine whether sulfation of the hydroxyl group of BI-2 or another benz[*e*]indene will result in compounds devoid of potentiation action but with more potent inhibitory action, as generally has been found for sulfated steroids acting at $GABA_A$ receptors.

Acknowledgments

We thank John Bracamontes for help with molecular biology and Charles F. Zorumski and Steven Mennerick for comments on the manuscript.

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