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The differential antagonism by bicuculline and SR95531 of pentobarbitone-induced currents in cultured hippocampal neurons

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Abstract

In voltage clamped cultured hippocampal neurons, application of γ-aminobutyric acid (GABA) or pentobarbitone induced chloride current in a dose-dependent manner. The dose dependence of these agonists were well described by ED_{50} and Hill coefficients of 14.7 ± 7 μM and 1.2 ± 0.5, and 299 ± 17.3 μM and 1.6 ± 0.1, for GABA and pentobarbitone, respectively. The effects of two $GABA_A$ receptor antagonists, bicuculline and 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyridazinium bromide (SR95531) were evaluated by coapplication of increasing concentrations of the antagonists with a fixed equipotent (approximately ED_{30}) dose of GABA or pentobarbitone. Both bicuculline and SR95531 blocked the GABA induced current with ID_{50} and Hill coefficients of 0.74 ± 0.07 μM and 0.96 ± 0.07, and 0.44 ± 0.02 μM and 1.22 ± 0.06, respectively. Bicuculline similarly blocked the pentobarbitone induced current with a ID_{50} and Hill coefficient of 0.69 ± 0.04 μM and 1.2 ± 0.1. However, pentobarbitone induced current was poorly blocked by SR95531 retaining 86.5% of current amplitude at a concentration of SR95531, 200 times the IC_{50} for GABA induced current. Current induced by etomidate, another intravenous general anesthetic with $GABA_A$ receptor agonistic property, is likewise resistant to SR95531 blockade. Three-dimensional modeling of bicuculline and SR95531 with alignment of the molecules along the suggested GABA-receptor binding moiety indicates that these two antagonist molecules have distinct steric properties. We suggest that GABA and pentobarbitone act at nearby but non-identical sites on the hippocampal $GABA_A$ receptor to open the chloride ionophore, and that these sites can be distinguished by bicuculline and SR95531. This is the first demonstration that the prototypic $GABA_A$ site antagonists bicuculline and SR95531 have different effects on currents induced by GABA and pentobarbitone.

Keywords: GABA receptor antagonist; Bicuculline; SR95531; Pentobarbitone; Etomidate; Hippocampal neuron

1. Introduction

The $GABA_A$ receptor is a member of the ligand gated ion channel superfamily and mediates inhibitory neurotransmission in the brain. Cumulative evidence suggests that the $GABA_A$ receptor possess five distinct functional sites: the GABA receptor agonist site, the benzodiazepine site, the steroid site, the barbiturate site, and the picrotoxin site (reviewed in Macdonald and Olsen, 1994; Smith and Olsen, 1995). It is well known that a variety of neuroactive drugs including general anesthetics, sedative/hypnotics, anticonvulsants, and neurosteroids directly open the ion channels or potentiate the action of GABA through effects on the $GABA_A$ receptors. While both radiolabeled biochemical and electrophysiological studies support the concept of a supramolecular $GABA_A$ receptor with a multitude of allosteric modulator and agonist binding sites, the precise molecular sites of action of these neuroactive drugs on the supermolecular complex remain unknown.

Pentobarbitone, a general anesthetic barbiturate, modulates the binding of radiolabeled GABA, benzodiazepines, and *t*-butylbicyclophosphorothionate (TBPS)/picrotoxin (Olsen, 1981; King et al., 1987; Bureau and Olsen, 1993). Electrophysiological experiments have shown that pentobarbitone and GABA cross-desensitize (Akaike et al., 1985), and currents induced by both drugs are blocked by the competitive $GABA_A$ receptor antagonist bicuculline (Akaike et al., 1987). This is consistent with the close proximity of the barbiturate site of action with the other

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functional sites on the receptor supramolecule, and in fact suggests that the barbiturate site may be identical to the GABA recognition site. However, recent experiments with expressed native and mutated GABA receptor subunit cDNAs show that pentobarbitone and GABA have different receptor amino acid requirements for their agonistic action, indicating that the sites of action may be distinct (Cutting et al., 1991; Amin and Weiss, 1993; Cestari et al., 1994). A clear pharmacological separation of the GABA and pentobarbitone action on the native $GABA_A$ receptor will clarify whether these two drugs act at the same or distinct sites.

Bicuculline, a phthalide isoquinoline alkaloid, is firmly established as a specific, competitive inhibitor of the $GABA_A$ recognition site (reviewed in Kerr and Ong, 1992). More recently, a series of pyridazinyl GABA-derivatives competitive $GABA_A$ antagonists have been synthesized (Wermuth and Biziere, 1986). Of these, the compound 2-(3-carboxypropyl)-3-amino-6-methoxyphenylpyridazinium bromide (SR95531) represents the derivative with optimal configuration for $GABA_A$ antagonism where the half-maximal inhibition for [^{3}H]GABA binding being two orders of magnitude less than bicuculline (Heaulme et al., 1986). In an investigation of the [^{3}H]muscimol interaction with specific GABA receptor polypeptide isolated from the brain, it was noted that bicuculline and SR95531 had different effects on some receptor polypeptides (Bureau and Olsen, 1993). This observation that these two $GABA_A$ competitive antagonists appear to distinguish the interaction of muscimol and the sites of action on different receptor polypeptides suggested to us that perhaps, these two competitive antagonists may be able to distinguish the interaction of GABA and pentobarbitone at their sites of action. Therefore, we conducted experiments on patch clamped cultured postnatal hippocampal neurons to investigate the ability of bicuculline and SR95531 to antagonize the chloride current induced by GABA or pentobarbitone. We show that while bicuculline antagonizes currents elicited by both compounds, SR95531 only antagonizes currents induced by GABA supporting the hypothesis that GABA and pentobarbitone act at distinct sites on the native hippocampal $GABA_A$ receptor polypeptide. We extend this distinct-site of action hypothesis to etomidate, another intravenous general anesthetic with the ability to induce current, and suggest that the $GABA_A$ receptor agonistic function of intravenous general anesthetics may be mediated by a site distinct from the GABA recognition site.

2. Materials and methods

2.1. Cell culture

Hippocampi obtained from 1–4 days old postnatal rat pups were mechanically dissociated by trituration alone in Ca^{2+} free Pucks solution and plated on a Matrigel (Collaborative Research) coated plastic cover slips placed inside a 35 mm tissue culture dish. The plating medium consisted of a high-glucose (4500 mg/l) Dulbecco modified minimum Eagle's media containing HEPES and 2 mM L-glutamine supplemented with 10% (v/v) fetal calf serum, 10% (v/v) horse serum, 50 μg/ml streptomycin and 50 U/ml penicillin. Cytosine-arabinoside (1 μM) was added 24–48 h after plating to suppress the growth of background cells, and thereafter, the media was changed to a serum-free plating medium supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), and selenium (10 ng/ml) (Sigma I1884). The postnatal hippocampal culture tolerated the defined media well with the neurons evenly dispersing over the confluent background glia cells. To optimize spatial clamping, cultures were used for electrophysiological experiments within 5 days after plating.

2.2. Whole cell patch clamp

Cells viewed under an inverted microscope equipped with Hoffman optics (Nikon TMS) were randomly selected and the whole cell mode of patch clamp established. In general, large cells with multipolar morphology indicative of pyramidal neurons were selected but morphological criteria alone is not sufficient to definitively distinguish the different neuronal cell types present in a mixed hippocampal culture (Yang et al., 1993). External solution which continuously perfused the recording chamber (1.5 ml total volume) consisted of (in mM): 140 NaCl, 4 KCl, 4 $MgCl_2$, 10 Hepes, 10 dextrose and pH titrated to 7.4 with 1 N NaOH. The action potential dependent synaptic currents were blocked by 0.5 μM tetrodotoxin added to the external medium. The patch electrodes fashioned from a 1.2 mm fiber-filled borosilicate glass capillary (WPI) were fire-polished and filled with an internal solution consisting of (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 10 Hepes, 10 EGTA, and pH titrated to 7.4 with 1 N CsOH. The junction potential was nulled with the electrode in the bath prior to contacting the cell and the bath was grounded to the amplifier (AI 200, Axon Instruments) via a Ag/AgCl agar-CsCl bridge. The charging curve for cultured hippocampal neurons up till 7 days in vitro remained monoexponential for over 75% of cells (Yang et al., 1993). In these cells, series resistance compensation was easily accomplished by the amplifier's analog compensation circuitry. The cell input capacitance was directly read from the amplifier knob. All experiments were done on neurons held at a transmembrane potential of −60 mV and at room temperature (20–23°C).

2.3. Drug application

Drugs at the desired concentrations were prepared in the external solution daily from frozen stock solutions of 100 mM GABA, 100 mM Na-pentobarbitone and 8.2 mM

etomidate. Application of propylene glycol, the vehicle for etomidate at concentrations $< 0.1\%$ had no effect on GABA gated currents. The drug application system consisted of a gravity driven U-tube system fashioned from a PE10 tubing connected to rotatory valve selective reservoirs. A U-tube orifice of approximately 200 μm was cut out using the bevel of a 27 G hypodermic needle under direct vision with a binocular scope. The downstream side of the U-tube was connected to a solenoid valve such that application of a +5 V transistor-transistor logic signal controlled by the computer would close the valve and expel the desired solution onto the cell via the U-tube orifice essentially as described by Shirasaki et al. (1991). Prior to data collection, the optimal position of the U-tube orifice was determined for each cell to ensure maximum response rate. The positioning was facilitated by adding a drop of phenol red to the drug solutions so that the stream of solution exiting the U-tube could be seen. The approximate exchange time determined from the change in the whole cell holding current upon application of a hyperosmotic solution was about 200 ms. While the relatively slow solution exchange time will miss fast desensitizing events and potentially bias the data, the rate of current desensitization for the $GABA_A$ receptors in hippocampal neurons is slow (Huguenard and Alger, 1986) resulting in minimal error. The timing of drug applications and data digitization was controlled by Pclamp v5.2 (Axon Instruments). The drug application interval was adjusted between 3–10 min to prevent accumulation of desensitization. Cumulative desensitization not reversed by 10 min application interval was particularly noticeable for concentrations of pentobarbitone greater than 200 μM. Longer application intervals resulted in an irreversible decline in the magnitude of the current response during the course of an experiment, which in our hands, was not prevented by including ATP in the intracellular solution (Gyenes et al., 1989). Therefore, 10 min intervals, based on a balance between cumulative desensitization and irreversible rundown of the current, were chosen as the maximum time interval between drug applications. The antagonists were dissolved in the external solution from stock solutions (100

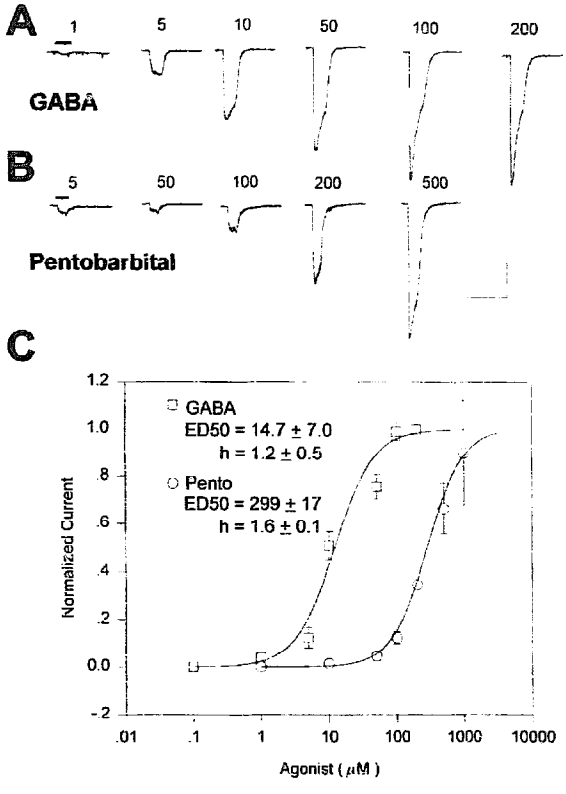


Fig. 1. GABA and pentobarbitone induce currents in a dose-dependent manner. Current responses of neurons voltage clamped at −60 mV exposed to increasing concentrations of GABA (A) or pentobarbitone (B). The drug application duration (5 s for GABA and 3 s for pentobarbitone) identical for all traces for a given drug is denoted by a solid bar over the left-most traces. The pentobarbitone application was shorter than for GABA to minimize accumulation of desensitization (see Methods). (C) Cumulative dose-response to GABA (open squares) and pentobarbitone (open circles). The current magnitudes were normalized by the I_{max} value obtained from the theoretical fit of the Hill equation which were 2800 pA and 2042 pA for GABA and pentobarbitone, respectively. Each symbols are based on 3–8 neurons and error bars represents the standard deviation. Calibration bars are 800 pA (for GABA), 400 pA (for pentobarbitone) and 17.5 s for both.

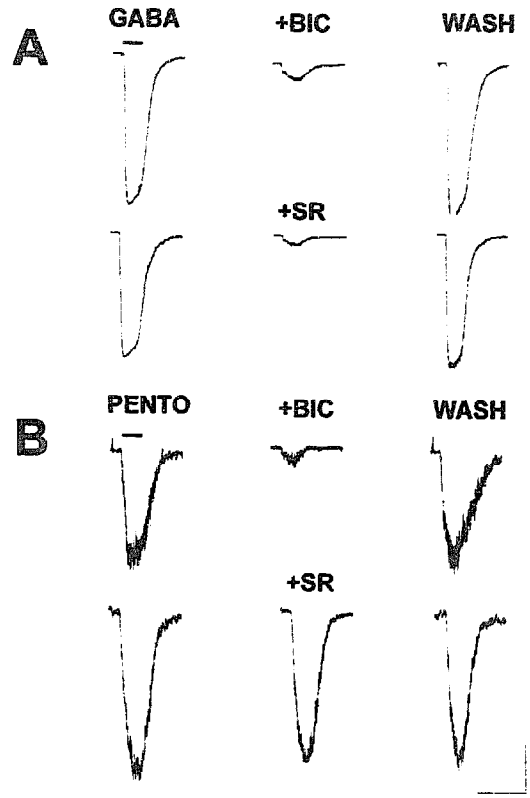


Fig. 2. Bicuculline and SR95531 show differential antagonism on GABA and pentobarbitone induced currents. Each set of three traces show the control (left), drug effect (middle), and washout (right) for 10 μM bicuculline (BIC) or 10 μM SR95531 (SR) on GABA (5 μM) (A) or pentobarbitone (150 μM) (B). The solid bar above the first traces indicates the duration of drug application. Calibration bars are 625 pA (A), 150 pA (B) and 17.5 s for both.

mM bicuculline methylbromide and 10 mM SR95531 both in water) and simultaneously applied with the agonist through the same U-tube orifice. Except for etomidate and SR95531 which were purchased from Abbott Laboratories and RBI, respectively, all other chemicals were purchased from Sigma.

2.4. Data analysis

The magnitude of current responses to drug applications was measured directly by cursors in Clampex. The collated responses were entered into SigmaPlot (Jandel Scientific) and the equation of the form $I/I_{max} = (1/(1 + ([drug]/ED_{50})^{**}h))$, where I_{max} = maximum current, ED_{50} = half-maximal dose, and h = Hill coefficient, fit by a non-linear function minimizing routine without constraints on the parameters. Averaged values are presented as means ± S.D. The statistical significance of the observations were determined by a two-sided Student's t-test.

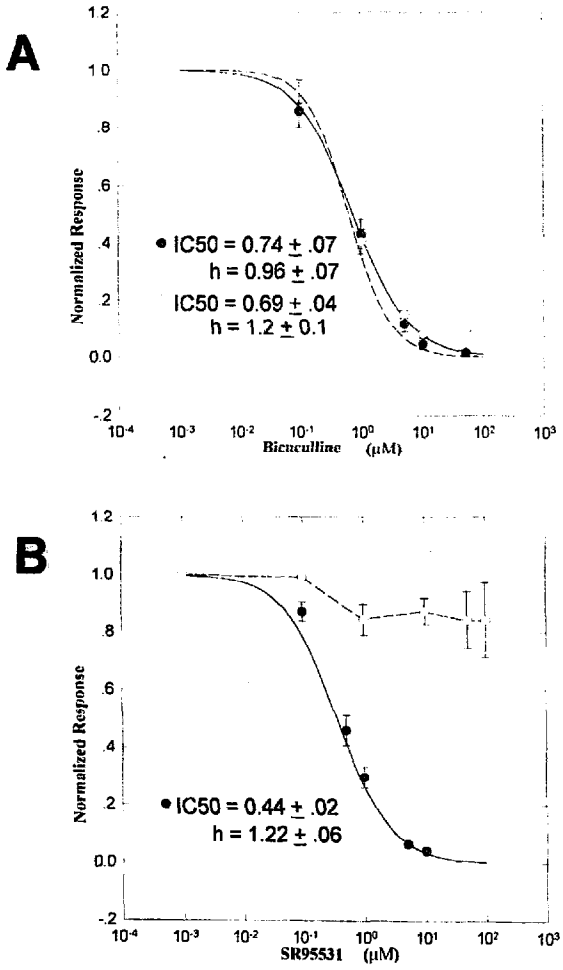


Fig. 3. Summary dose response of bicuculline and SR95531 inhibition. Cells were exposed to a fixed 5 μM GABA (solid circles) or 150 μM pentobarbitone (open circles) dose, and increasing concentrations of bicuculline (A) or SR95531 (B) were coapplied. Individual response magnitudes were normalized to the control response, collated, and plotted as the normalized response. The solid curves are the best-fitting curve determined by fitting a Hill equation of the form $I = (1 - ([D]^{**}h/[D]^{**}h + ID_{50}^{**}h))$ where ID_{50} is the concentration of the antagonist ([D]) at half-maximal blockade, and h = Hill coefficient. Each symbol represents data from 5 different cells.

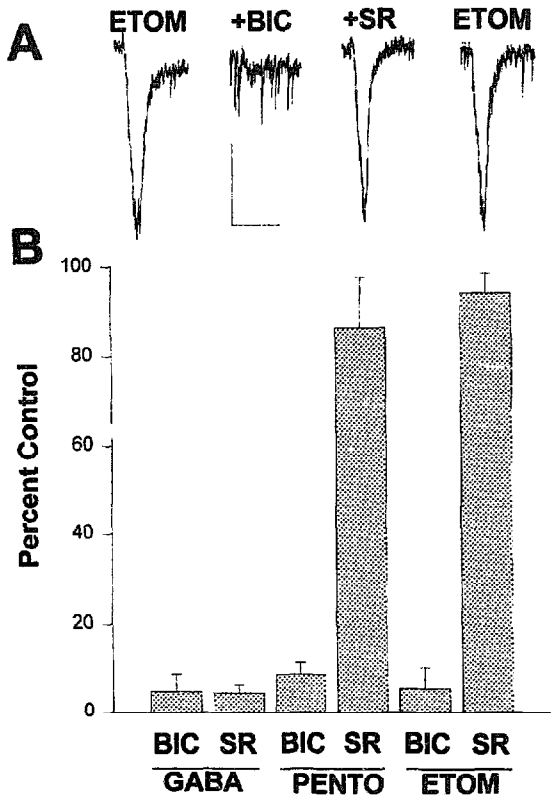


Fig. 4. SR95531 fails to block the etomidate induced current. (A) Current traces to 100 μM etomidate application alone (left), with 10 μM bicuculline or SR95531 (middle traces), and again by itself (right). A complete dose-response to etomidate could not be obtained because of irreversible run-down of the current at etomidate concentrations > 1 mM. The 100 μM dose of etomidate probably reflects a value closer to ED_{10} compared to the ED_{30} doses used for pentobarbitone and GABA. The effect of using a lower agonist concentration is an over-estimation of the antagonist potency. The calibration bars are 100 pA and 17.5 s. (B) A bar graph summary comparing bicuculline (BIC) and SR95531 (SR) effect on currents induced by GABA, pentobarbitone, and etomidate. Summary based on 4–6 cells exposed to 5 μM GABA, 150 μM pentobarbitone, or 100 μM etomidate.

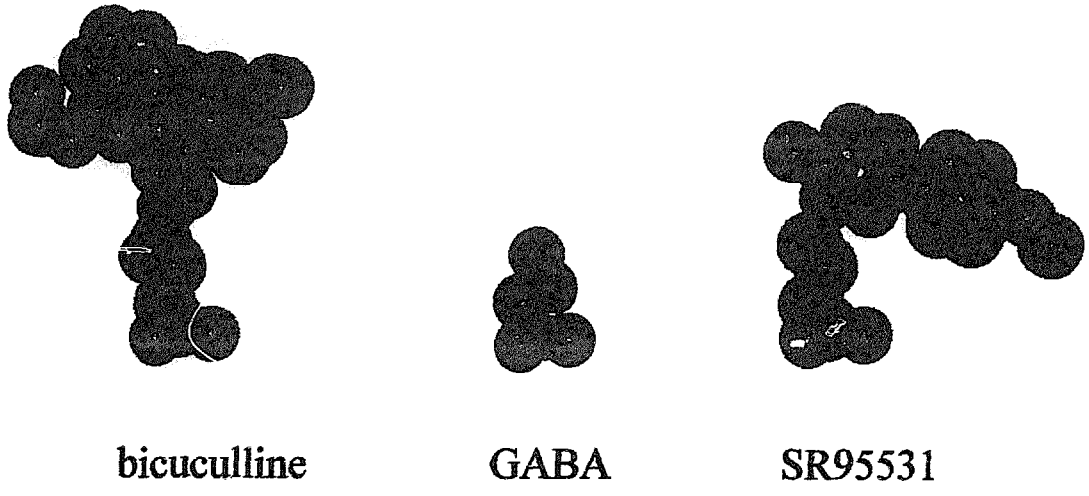
2.5. Molecular modeling

The 3-dimensional molecular modeling of bicuculline methylbromide, SR95531 and GABA was done using HyperChem v 4.0 (Hypercube, Ont., Canada) running under Windows on a 486 DX2/66 MHz personal computer. The optimum geometry of the molecules in an aqueous environment was determined by energy minimization of a periodic box (18.7 angstrom cube) filled with water molecules and the desired compound. The energy minimization was accomplished by the Polak-Ribiere algorithm using the MM + force field ('Using Molecular Mechanics', in: Computational Chemistry, HyperChem Inc., Waterloo, Ont., Canada, pp. 83–88) and the resulting energetically

optimum molecular conformations were rotated in space to visually align the GABA-like moiety of each compound.

3. Results

Extracellular applications of GABA or pentobarbitone induced dose-dependent inward currents in neurons voltage clamped at negative holding potentials (Fig. 1). At high agonist concentrations, the current magnitude declined significantly during the drug application period due to desensitization. Dose dependence of the peak current amplitude was well described by a Hill equation with concentration for half-maximal response (ED_{50}) and Hill coefficient (h) of $ED_{50} = 14.7 \pm 7$ μM, $h = 1.2 \pm 0.5$, and $ED_{50} = 299 \pm 21$ μM, $h = 1.6 \pm 0.1$, for GABA and pen-



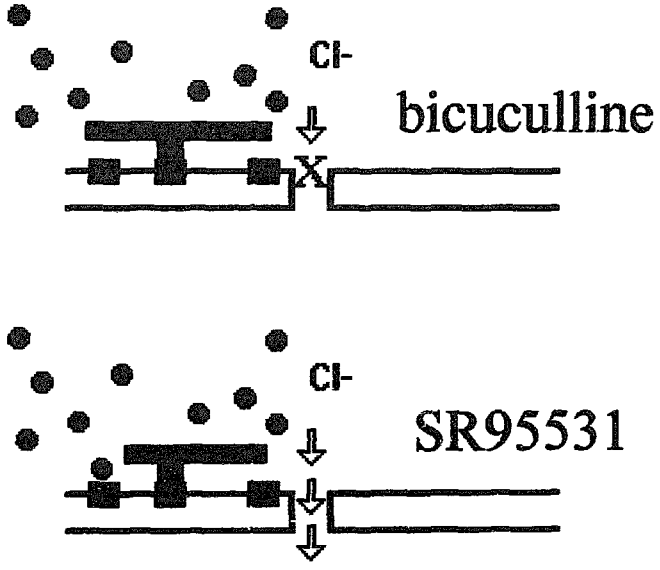


Fig. 5. Molecular modeling of bicuculline, GABA, and SR95531. (A) Space-fill renditions of the energetically optimum conformation of the three molecules were aligned with the GABA-like moiety positioned at the bottom of the molecules. Along this particular axis of view, SR95531 extends the aromatic portion of the molecule largely to its right, whereas, the bulky bicuculline molecule occupies space in both directions. The atoms are color-coded as: red (oxygen), blue (nitrogen), and aqua (carbon). Hydrogen atoms have been deleted for clarity. (B) A hypothetical model showing differential action of bicuculline and SR95531 on ion channel opening by GABA and pentobarbitone. Assuming that both bicuculline and SR95531 are binding to the same site (red square) on the receptor macromolecule, the sterically larger bicuculline blocks both the GABA (blue circles) and pentobarbitone (green circles) access to their respective sites-of-action (top). The sterically more compact SR95531 blocks GABA access to its site-of-action, but not that of pentobarbitone (bottom). In this illustration, the antagonist binding site is designated as a distinct site from the GABA binding site as has been demonstrated for strychnine on the glycine receptor (Vandenberg et al., 1992). However, the antagonist binding site could be identical to the GABA binding site similar to the nicotinic acetylcholine receptor (see review by Galzi et al., 1991).

tobarbitone, respectively. The maximum currents elicited normalized to the cell input capacitance were 112 ± 21 pA/pF and 78.4 ± 17 pA/pF for GABA and pentobarbitone, respectively. Since normalization by cell input capacitance compensates for the possible difference in the current magnitude due to a chance difference in the cell size, the smaller normalized current elicited by pentobarbitone indicates that pentobarbitone is a partial agonist of GABA. Tail currents developing only on drug washout were seen for pentobarbitone concentrations > 1 mM previously described for frog sensory neurons (Akaike et al., 1987), but were not pursued further. The GABA ED_{50} and h obtained here agrees well with 6.4 μM and 1.1 (Shirasaki et al., 1991) or 8.2 μM and 1.8 (Hara et al., 1994) obtained from acutely dissociated 1–2 week old rats using a similar Y-tube method of drug application, and to 19 μM and 1.2 obtained from cultured mouse hippocampal neurons using a multibarrel method of drug application (Orser et al., 1994).

To properly investigate the effects of competitive antagonists on two different agonists, equipotent doses of the agonists must be used. Based on the dose response obtained above, an approximate ED_{30} concentration of GABA (5 μM) and pentobarbitone (150 μM) was chosen. Fig. 2 shows the effects of 10 μM bicuculline or SR95531 coapplied with either GABA (A) or pentobarbitone (B). GABA induced currents were reversibly blocked by both bicuculline and SR95531. In contrast, pentobarbitone induced currents were only blocked by bicuculline. Fig. 3 summarizes the results from neurons exposed to increasing concentrations of the antagonists with a fixed ED_{30} dose of the agonist. Bicuculline was equipotent against both GABA (IC_{50} = 0.74 ± 0.07 μM, h = 0.96 ± 0.07) and pentobarbitone (IC_{50} = 0.69 ± 0.04 μM, h = 1.2 ± 0.1). SR95531 similarly blocked GABA induced currents (IC_{50} = 0.44 ± 0.02 μM, h = 1.22 ± 0.06), but failed to block the pentobarbitone induced current. At 100 μM which was the highest concentration of SR95531 tested, pentobarbitone current persisted at 86.5 ± 14% of control.

Etomidate is a non-barbiturate intravenous general anesthetic with GABA-modulatory and GABA-agonistic properties similar to barbiturates. Therefore, to examine whether the differential blocking property between bicuculline and SR95531 was unique to pentobarbitone induced current, the effect of these antagonists was examined on etomidate. Etomidate at micromolar concentrations induced currents in the absence of GABA (Evans and Hill, 1978). Fig. 4A shows current responses to 100 μM etomidate. Coapplication of 10 μM bicuculline completely abolishes the response whereas, SR95531 at the same concentration has no effect. Fig. 4B summarizes the effect of a fixed dose of bicuculline and SR95531 on GABA, pentobarbitone, and etomidate induced currents. The lack of SR95531 blockade of etomidate is identical to pentobarbitone.

In order to gain insight into the possible difference in the mechanism of action of bicuculline and SR95531, a 3-dimensional conformation of these molecules was determined by molecular simulation. Since these bulky compounds act on the aqueous extracellular surface of the transmembrane receptor protein, the conformation of the molecules was energetically optimized for an aqueous environment. Fig. 5A shows a space-fill rendition of bicuculline, GABA, and SR95531 with the putative GABA recognition regions of the molecules (Kerr and Ong, 1992; Rognan et al., 1992) aligned with the GABA molecule itself. Along this axis of view, the conformationally rigid aromatic extension of SR95531 extends to the right, while bicuculline extends to either side of the GABA recognition region. The two antagonists clearly occupy distinct space and could provide differential steric hindrance to spatially close but distinct sites (Fig. 5B).

4. Discussion

Our data demonstrate that in cultured hippocampal neurons, the agonistic action of pentobarbitone and GABA is distinguishable by the two prototypic $GABA_A$ receptor antagonists bicuculline and SR95531. Extensive molecular modeling of $GABA_A$ receptor antagonists, including bicuculline and SR95531, have identified a cationic and an anionic binding sites separated by 4.6–5.2 A as a common structural feature (Rognan et al., 1992). Therefore bicuculline and SR95531 are thought to bind to identical sites (or at the least partially overlapping sites) on the receptor macromolecule. Given this reasonable asumption, there are two possible reasons for the selective inability of SR95531 to block the pentobarbitone induced current. The first possibility is that GABA and pentobarbitone act at nearby, but distinct sites and the more spatially compact SR95531 fails to provide steric hindrance to prevent pentobarbitone access to its site. The second possibility is that GABA and pentobarbitone each only activate a subpopulation of receptor isoforms and SR95531 is inactive at the isoform responsive to pentobarbitone.

The most convincing data to date which suggest distinct sites of action for GABA and pentobarbitone comes from an electrophysiological study of expressed $GABA_A$ receptor subunit cRNAs (Amin and Weiss, 1993). In this study, amino acids in the extracellular region of the GABA receptor β_2-subunit were mutated, and the effect on GABA and pentobarbitone dose response investigated. Two separate and homologous domains of tyrosine and threonine on the β_2 subunit were identified which when mutated, shifts the GABA affinity for its site by 2 orders of magnitude. However, these mutants had no effect on the pentobarbitone dose response. This clear difference in the amino acid requirements for GABA and pentobarbitone induced currents strongly argues for distinct sites of action, at least in expressed heteroligomeric $GABA_A$ receptors of a defined subunit composition. It is not known whether the agonist property of other non-barbiturate compounds with GABA-

receptor agonistic properties such as etomidate, propofol and neurosteroids are altered in these mutants with a low affinity for GABA. Such a dissociation between GABA and general anesthetic's ability to induce current is well documented for the $GABA_C$ receptor subunit $\rho 1$. This subunit which is predominantly found in the retina forms a functional homoligomeric receptor opened by GABA (Cutting et al., 1991). The general anesthetics pentobarbitone or isoflurane do not induce currents by themselves, or potentiate current induced by GABA (Shimada et al., 1992; Harrison et al., 1993) in $\rho 1$ homoligomers. A converse situation where general anesthetics (pentobarbitone, etomidate, and propofol) induce current but GABA fails to open ion channels is observed for murine β_3 subunit homoligomers (Cestari et al., 1994). Clearly, in these simple homoligomeric receptors, GABA and general anesthetics are acting at different sites. Our present data on hippocampal neurons suggest that distinct sites of action for GABA and pentobarbitone, and possibly other general anesthetics, may be true for native $GABA_A$ receptors as well.

An alternative interpretation of the differential blockade of GABA and pentobarbitone induced currents by bicuculline and SR95531 is that the respective agonists may be selectively acting on a subpopulation of heterogeneous receptor subtypes found in neurons. According to this hypothesis, the receptors responsive to GABA are blocked by both bicuculline and SR95531, whereas the subpopulation responsive to pentobarbitone is only sensitive to bicuculline. Even within a confined brain area such as the hippocampus, it is clear that several $GABA_A$ receptor subunits are present (Wisden et al., 1992; Fritschy et al., 1992) and the receptor subunit composition varies during development (Laurie et al., 1992). These subunits could combine in a number of permutations to give rise to a heterogeneous receptor population with different pharmacological properties. The presence of subpopulation of $GABA_A$ receptors with differential sensitivity to the two prototypic $GABA_A$ competitive antagonists is consistent with the observation by Bureau and Olsen (1993) that the 51 kD and 53 kD [^{3}H]muscimol binding proteins isolated from the rat brain show differential sensitivity to bicuculline and SR95531. On the other hand, receptor subunit mix-and-match experiments failed to identify subunit specificity of the direct current induced by pentobarbitone (Sigel et al., 1990), or pentobarbitone potentiation of GABA induced current (Horne et al., 1993; Puia et al., 1990; Levitan et al., 1988). This is in contrast to the well documented receptor subunit dependence of the benzodiazepine and β-carboline modulation of the GABA gated current (Pritchett et al., 1989; Puia et al., 1991; Wafford et al., 1993). Furthermore, no known heteroligomeric combination of receptor subunits directly responsive to pentobarbitone fails to respond to GABA (Sigel et al., 1990), and the presence of such receptor subpopulation is essential for the selective activation hypothesis to be valid. Therefore it is unlikely that a difference in the target subpopulation where GABA and pentobarbitone act upon is at the basis for the divergence between bicuculline and SR95531 blockade. However, we can not rule out the possibility that a particular subunit composition, perhaps expressed in early development, may selectively respond to pentobarbitone. The inability of SR95531 to block current induced by another intravenous general anesthetic etomidate suggests that the site of action of etomidate may also be distinct from the GABA recognition site. It remains to be seen whether etomidate and pentobarbitone occupy a mutually exclusive site of action but given the pharmacological similarity in their effects on the $GABA_A$ receptor (Evans and Hill, 1978; Proctor et al., 1986), an identical site of action for these two agents is a strong possibility.

In conclusion, we provide pharmacological support to the idea that GABA and pentobarbitone act at nearby but distinct sites to open the chloride ionophore and extend this concept to the native hippocampal $GABA_A$ receptor. The structurally bulkier bicuculline when bound to its site protects both the GABA and pentobarbitone binding sites resulting in competitive antagonism for both. The more compact SR95531 occupies a mutually exclusive space only with GABA. Given the reasonable assumption that both bicuculline and SR95531 bind to an identical site, a hypothesis is proposed that the GABA binding site is spatially closer to the antagonist binding site than the pentobarbitone binding site. Unfortunately, how the difference in the physical dimensions of spheres of steric hindrance translates into physical sites of GABA and pentobarbitone actions on the receptor polypeptide can not be determined because of the undoubtedly complex protein folding. Further investigation of differential pharmacological actions of bicuculline and SR95531 on GABA receptor site-directed mutants is needed to precisely define the molecular site of action of pentobarbitone and other presumed $GABA_A$ receptor agonists.

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