Selection of Stable RNA Molecules that Can Regulate the Channel-Opening Equilibrium of the Membrane-Bound γ-Aminobutyric Acid Receptor†

Yang Cui, Priyamvada Rajasethupathy, and George P. Hess*

Department of Molecular Biology and Genetics, 216 Biotechnology Building, Cornell University, Ithaca, New York 14853-2703

Received June 25, 2004; Revised Manuscript Received September 16, 2004

ABSTRACT: The γ-aminobutyric acid ($GABA_A$) receptor belongs to a superfamily of membrane-bound proteins that regulate signal transmission between cells in the nervous system. It is the target of convulsants such as picrotoxin and is mutated in some forms of epilepsy, a disease affecting ~50 million people worldwide. In picrotoxin inhibition and in one form of epilepsy, a decrease in the channel-opening equilibrium of a $GABA_A$ receptor is responsible for receptor dysfunction. Here we identify compounds that can regulate the channel-opening equilibrium of the $GABA_A$ receptor. Fluorinated RNA polymers containing a 40-nucleotide region with a randomized sequence were used to select those that can displace picrotoxin from the membrane-bound $GABA_A$ receptor in the rat forebrain. After 11 selection rounds, two classes of RNA molecules that bind to the $GABA_A$ receptor with nanomolar affinity were isolated and sequenced. Class I and class II molecules have different consensus sequences and different binding affinities for the receptor. A transient kinetic technique, the cell-flow method, was employed in combination with the whole-cell current-recording technique to determine the affinity of the selected RNA aptamers for the $GABA_A$ receptor. Class I molecules have a higher affinity for the closed-channel form than for the open-channel receptor form and inhibit the receptor; class II aptamers bind with equal or higher affinity to the open-channel form and alleviate picrotoxin inhibition.

$GABA_A$[1] receptors belong to a superfamily of membrane proteins that regulate intercellular signal transmission between approximately 10^{12} cells of the mammalian nervous system (*1*). They mediate a majority of the inhibitory neurotransmission pathways in the mammalian brain and control the flux of chloride ions across the cell membrane (*2*). Various drugs allosterically modulate this chloride ion flux (*3*). Evidence has accumulated correlating some forms of epilepsy with dysfunction of the GABA-ergic neurons (*4*, *5*). A decrease in the rate constants for channel opening and closing, as well as a decrease in the affinity for GABA, has been reported in genetically epilepsy-prone rats (*6*). A single mutation in the $\gamma 2$ subunit of a human $GABA_A$ receptor linked to a generalized epilepsy with febrile seizures plus (GEFS+) leads to a significantly weakened response of the receptor to GABA (*7*). This dysfunction has been shown to be due to a greatly reduced channel opening equilibrium (*8*). Although several anticonvulsant drugs have been developed (*5*, *9*), the extent to which they counteract a dysfunctional $GABA_A$ receptor is not known.

Picrotoxin, a known convulsant that induces seizures, plays an important role in studies of antiepileptic drugs and $GABA_A$ receptors (*10*). Reports indicate that many convulsants, anticonvulsants, sedatives, hypnotics, and anxiolytics exert their characteristic effects by acting on the picrotoxin-binding site of $GABA_A$ receptors (*11*). Interestingly, alkyl-substituted γ-butyrolactones (GBLs) and γ-thiobutyrolactones (TBLs), which are neuroactive chemicals that have convulsant or anticonvulsant activity depending on the location of the alkyl constituents (*12*), act on the same site on the $GABA_A$ receptor as picrotoxin (*13*).

It has recently been demonstrated that picrotoxin, which causes convulsions in animals, inhibits the $GABA_A$ receptor by decreasing its channel-opening equilibrium (*14*). The model for the inhibition of the nicotinic acetylcholine receptor (nAChR) by cocaine (*15*) is shown in Figure 1. It is also pertinent to the inhibition of the $GABA_A$ receptor by picrotoxin and the alleviation of this inhibition (*14*).

The reactions shown in Figure 1 occur in the microsecond-to-millisecond time region (*16*−*20*). For clarity, the desensitization reaction, which in the case of the nAChR occurs in the 100−500 ms time region (*20*, *21*), and the binding of inhibitor I to the unliganded receptor form are not shown. The proposed cyclic inhibition mechanism in Figure 1 involves a complex of the inhibitor with the open-channel conformation in which the open channel is not blocked by the inhibitor (i.e., it conducts ions). This minimum mechanism of inhibition of the receptor is based on chemical kinetic measurements. It makes predictions regarding the properties of ligands that will inhibit the receptor and of those that will not inhibit the receptor but will prevent the binding of noncompetitive inhibitors (*15*). The principle of microscopic reversibility (*22*) requires that the $\bar{K}_I/K_I$ ratio equal Φ_{I0}^{-1}/Φ^{-1}, where $\Phi^{-1} = k_{op}/k_{cl}$ and $\Phi_{I0}^{-1} = k^*_{op}/k^*_{cl}$ (Figure 1).

† This work was supported by a grant (NS08527) awarded to G.P.H. by the National Institutes of Health, National Institute of Neurological Disease and Stroke.

* To whom correspondence should be addressed. Telephone: (607) 255-4809. Fax: (607) 255-6249. E-mail: gph2@cornell.edu.

[1] Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; $GABA_A$, γ-aminobutyric acid; SELEX, systematic evolution of ligands by exponential enrichment; nt, nucleotide; PCP, 1-phenylcyclohexylpiperidine; TBPS, *tert*-butylbicyclophosphorothionate; TBOB, *tert*-butylbicycloorthobenzoate; nAChR, nicotinic acetylcholine receptor.

10.1021/bi048667b CCC: $27.50
Published on Web 11/25/2004

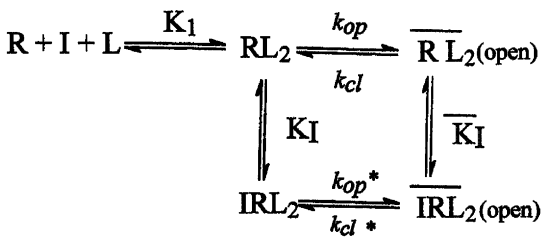

FIGURE 1: Proposed mechanism for the inhibition of the nAChR by cocaine. The upper line represents the minimum mechanism for the opening of the receptor channel (*47*). Receptor R binds neurotransmitter L. Katz and Thesleff (*47*) first suggested the binding of at least two ligand molecules to the receptor before the receptor channel opens. R, RL, and RL_2 represent the closed-channel conformations. $\overline{RL_2}$ represents the open-channel conformation of the receptor that allows small inorganic ions to cross the cell membrane, thus initiating an electrical signal and intercellular communication. K_1 is the observed dissociation constant for the activating ligand. k_{op} and k_{cl} are the rate constants for channel opening and closing, respectively. Φ^{-1} ($=k_{op}/k_{cl}$) is the channel-opening equilibrium constant (*45*, *48*).

Therefore, compounds that bind to a regulatory site with higher affinity for the closed-channel conformation than for the open-channel form will shift the equilibrium toward the closed channel form and inhibit the receptor. Compounds that bind to the open-channel conformation with equal or higher affinity than to the closed-channel form are expected not to decrease the channel-opening equilibrium. They can, therefore, displace inhibitors from the regulatory site without inhibiting receptor activity. In the case of the nAChR, it was demonstrated (*23*) that RNA ligands (aptamers) can be found, using the power of combinatorial synthesis (*24*, *25*), that bind to the same regulatory site as cocaine and other noncompetitive inhibitors. Those aptamers fall into two classes. Members of one class with a certain consensus sequence bind with higher affinity to the closed-channel receptor than they do to the open-channel form and inhibit the receptor (*15*, *23*). Members of the second class, with a different consensus sequence, bind with equal or higher affinity to the open-channel form, do not inhibit the receptor but do prevent noncompetitive inhibitors such as cocaine from binding (*15*). Similarly, our objective in the experiments described here was to determine if RNA ligands can be found that can counteract the decrease in the channel-opening equilibrium of the $GABA_A$ receptor induced by picrotoxin (*14*). The results presented here indicate that such RNA aptamers exist; electrophysiological measurements demonstrated they bind with higher or equal affinity to the open-channel receptor form than to the closed channel form. We demonstrate that these RNA aptamers can alleviate picrotoxin inhibition of the $GABA_A$ receptor expressed in HEK 293 cells (see Figure 4). It is hoped that a similar approach using the mutated $GABA_A$ receptor linked to epilepsy (*7*) can be used to alleviate its unfavorable channel-opening equilibrium constant (*8*).

To select ligands (RNA aptamers) that compete with picrotoxin for its binding site on the $GABA_A$ receptor, RNA polymers containing a 40-nucleotide region with a completely randomized sequence in combination with an *in vitro* selection method known as the systematic evolution of ligands by exponential enrichment (SELEX) (*24*, *25*) were used. This method with modifications can be used with membrane-bound proteins (*23*). Furthermore, the modification of natural RNAs with fluoro groups at the 2′-hydroxy position in the pyrimidine nucleotides was used to generate nuclease-resistant random oligonucleotide libraries (*26*, *27*). 2′-Fluorodeoxypyrimidine triphosphates can be incorporated into RNA by T7 RNA polymerase in the SELEX steps (*28*). In this report, we describe the selection and characterization of 2′-fluoro-modified RNA ligands for the picrotoxin-binding site of the $GABA_A$ receptor.

EXPERIMENTAL PROCEDURES

Materials. Sprague-Dawley rats of either sex, 30−90 days old, were purchased from Charles River (Kingston, NY) for membrane preparations. Picrotoxin, GABA, and other chemicals were purchased from Sigma. [^{3}H]-*tert*-Butylbicycloorthobenzoate ([^{3}H]TBOB, 20.0 Ci/mmol), [^{32}P]ATP, and [^{32}P]UTP were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). 2′-Fluoro-2′-deoxycytidine 5′-triphosphate and 2′-fluoro-2′-deoxyuridine 5′-triphosphate were synthesized by TriLink BioTechnologies (San Diego, CA). The sources of other materials are noted in the relevant section.

Preparation of $GABA_A$ Receptor Membranes and Determination of Receptor Concentration. Membrane fragments containing the $GABA_A$ receptor with the picrotoxin site were prepared as described by Squires and Saederup (*29*, *30*). Sprague-Dawley rats of either sex, 30−90 days old, were euthanized with CO_2 and decapitated. The brains were removed, and the forebrain tissue was collected, weighed, and homogenized (Teflon glass) in 50 volumes (w/v) of ice-cold 2 mM ethylenediaminetetraacetic acid (EDTA)-Tris that was adjusted to pH 8.5 with 5 N sodium hydroxide. A crude membrane fraction was prepared by conventional differential centrifugation of the suspension at 2000*g* for 5 min at 4 °C. The supernatant was then centrifuged at 25000*g* for 30 min at 4 °C. The pellet was immediately resuspended in 20 volumes of 5 mM EDTA-Tris (pH 8.5) containing 200 mM NaCl and incubated for 15 min at 25 °C. This suspension was then diluted 1:1 with cold doubly deionized water (8 °C) before dialysis using a Spectra-Por 2 dialysis membrane (50 mm) 12−14000 MW cutoff (Fisher Scientific) that was soaked in 2 mM EDTA-Tris buffer (pH 8.5) overnight before use. The membrane suspension in the dialysis bag was then dialyzed, with magnetic stirring, against two successive portions of doubly deionized water for 2 h each. The volume of water outside the dialysis bag was ∼25 times the volume of the membrane suspension inside the bag. After high-speed centrifugation (25000*g*) at 4 °C for 30 min, the supernatants were discarded and the pellets frozen at −20 °C in plastic centrifuge tubes (∼50 mL capacity). The frozen pellet was resuspended in human embryonic kidney (HEK) 293 cell external buffer [145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, and 10 mM HEPES (pH 7.4)] before being used at a final protein concentration of 8−12 mg/mL. The membrane protein concentration was determined by the method of Lowry *et al.* (*31*). To remove endogenous GABA that has been a major impediment in determining how well [^{3}H]GABA, [^{3}H]muscimol, [^{35}S]TBPS, and [^{3}H]-TBOB bind to the membrane preparation, the buffer used for homogenization was adjusted to pH 8.5, and the pellets were stored at −80 °C (R. F. Squires, personal communica-

tion). These steps proved to be crucial in the completion of the project.

Selection of Stable Aptamers by SELEX. DNA Template and PCR Primer Synthesis. An 87-nucleotide oligonucleotide containing a 40-nucleotide region with a completely randomized sequence (*32*) flanked by two constant regions, and two primers, were synthesized by Keystone Labs, Biosource International (Camarillo, CA). The sequence of the two constant regions was 5′-GCC-TGT-TGT-GAG-CCT-CCT-GTC-GAA-40N-TT-GAG-CGT-TTA-TTC-TTG-TCT-CCC-3′, where N represents the random nucleotides (A, T, C, and G). The sequences of the two primers for extension and amplification were as follows: primer 24, 5′-GCC-TGT-TGT-GAG-CCT-CCT-GTC-GAA-3′; and primer 40, 5′-TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-AAG-AAT-AAA-CGC-TCA-A-3′.

The basic SELEX procedure was performed according to methods described previously (*23*−*25*). Briefly, RNA molecules are isolated and enriched from a large number ($\sim 10^{13}$) of combinationally synthesized RNAs by successive rounds of binding, displacement (by picrotoxin in this case), reverse transcription, RT-PCR, and transcription. In this way, the RNA pool with randomized sequences is enriched with the desired property. *In vitro* transcription with modified nucleotides (F-UTP and F-CTP) (*32*) created a fluoro-modified RNA pool that was used for selecting RNA aptamers that bind to the picrotoxin site of the $GABA_A$ receptor. The pool of modified RNA molecules in HEK 293 external buffer (pH 7.4) was denatured (5 min at 65 °C) and cooled to 4 °C. This pool containing 50 μM RNAs with randomized sequences was then incubated with extracted membrane fragments (0.8−1.2 mg of fresh rat forebrain tissue/100 μL) containing the $GABA_A$ receptor protein for 40 min at room temperature in a total volume of 500 μL of HEK 293 external buffer to enable the RNA to bind to the membrane-bound protein. The reaction mixture was passed through a filter holder with a 25 mm diameter nitrocellulose membrane disk (Schleicher & Schuell, Keene, NH). After three washes with 5 mL of external HEK buffer, the membrane protein−RNA complex retained on the filter was incubated with 100 μL of a 200 μM picrotoxin solution for 60 min, with gentle shaking to displace the RNA from the picrotoxin-binding site on the receptor. The solution containing eluted RNA specific for the picrotoxin-binding site was collected by centrifugation at 5000*g* for 10 min at 4 °C. The solution containing the displaced RNA was then extracted with a 1:1 volume of phenol and chloroform equilibrated with 0.1 M sodium acetate (pH 5.2) and chloroform. The product was precipitated with 3 volumes of absolute ethanol. The displaced RNA was amplified by RT-PCR (*24*). One step of reverse transcription PCR (RT-PCR) was performed by using Superscript one-step RT-PCR with the Platinum Taq system (Invitrogen, Carlsbad, CA) after each selection round to regenerate DNA from the selected 2′-F-RNA pool. The 50 μL reaction solution contained a 25 μL solution of 0.4 mM dNTP (dATP/dCTP/dGTP/dTTP mix), 2.4 mM $MgSO_4$, 1 μL of 100 μM primer 40, 1 μL of 100 μM primer 24, 1 μL of RT/Platinum Taq Mix, and 0.1−1 μg of 2′-F-RNA. The final volume was brought to 50 μL with nuclease-free dH_2O (Ambion). The cDNA synthesis was achieved using this reaction mixture during a 30 min incubation at 55 °C. The subsequent PCR was carried out for 10−15 cycles as follows: denatured at 94 °C for 1 min, annealed at 60 °C for 30 s, and extended at 72 °C for 1 min. The final extension cycle was at 72 °C for 10 min.

Determination of the Binding Affinity of the RNA Pool and of Individual Cloned Aptamers. Because of the availability of radioactive [^{3}H]TBOB (*tert*-butylbicycloorthobenzoate), which binds to the picrotoxin-binding site on the $GABA_A$ receptor (*33*−*35*), we used it to follow the selection of RNA aptamers that are displaced from the picrotoxin site of the $GABA_A$ receptor at different rounds of the procedure. The displacement of [^{3}H]TBOB by both picrotoxin and selected RNAs was measured. A fixed concentration (20 nM) of [^{3}H]-TBOB was used for displacement experiments in the presence or absence of the selected RNA, aptamers, and/or picrotoxin (Figure 2).

Cloning and Sequencing of the Selected RNAs. Selection rounds were terminated when the binding affinity of the RNA selection pool had remained constant for two rounds. The freshly purified PCR product was cloned using the Invitrogen pCR-4 TOPO TA cloning kit. This kit allows one to directly insert the Taq polymerase-amplified PCR product into a pCR-4 plasmid vector for sequencing. According to the protocol, the cloning reactions were transformed into Invitrogen One-Shot TOP10 competent *Escherichia coli* cells (Invitrogen protocol), and the transformed cells were plated and incubated at 37 °C. After 24 h, 100 colonies were selected and cultured in Luria-Bertani medium, and the plasmids were extracted and purified, and then sequenced by the Cornell University Biotechnology Resource Center's sequencing facility. RNA secondary structures were predicted using MFOLD (http://bioinfo.math.rpi.edu/~infold/rna/form1.cgi).

Characterization of Individual Cloned RNA Aptamers by Electrophysiological Techniques. The individual cloned RNA aptamers were also characterized by using an electrophysiological technique, whole-cell-current recording (*36*) in combination with the cell-flow technique (*17*). The cell-flow technique used to equilibrate cell surface receptors with ligands in the millisecond time domain has been described in detail (*17*, *19*, *37*, *38*). The cell-flow method includes a correction of the maximum amount of current observed at a specific GABA concentration. This correction takes into account the rapid desensitization that occurs while activating ligands equilibrate with the receptors on a single cell suspended by the current-recording electrode (*17*). A GABA solution flowed over a HEK 293 cell (WS-1) (American Type Culture Collection, Manassas, VA) expressing the $GABA_A$ receptor with $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits, and the induced cell current was recorded using the whole-cell-current configuration (*36*). Adding a selected aptamer to the flowing solution resulted in decreased or increased current amplitudes. Measurements were made by varying the concentration of a selected aptamer in the presence of GABA and in the presence or absence of picrotoxin to characterize the effect of the selected aptamer on the mechanism of the receptor-mediated reaction. All the cloned aptamers were tested by using the cell-flow technique (*17*) in combination with whole-cell-current recordings (*36*); the aptamers that did not inhibit the receptor were further tested for alleviation of picrotoxin inhibition.

The equations pertinent to the analysis of the electrophysiological measurements are given in the Appendix.

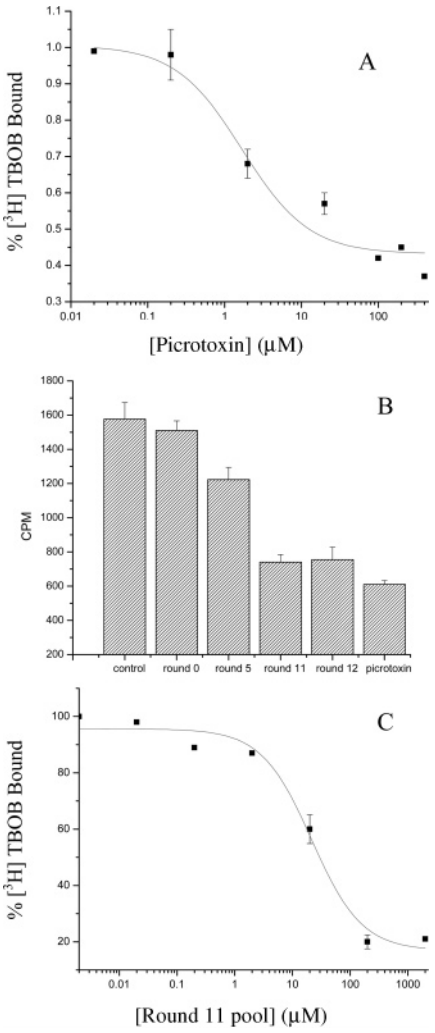

FIGURE 2: Displacement of 20 nM [^{3}H]TBOB bound to membrane fragments containing the $GABA_A$ receptor picrotoxin-binding sites by picrotoxin and by the enriched 2′-F-RNA pools obtained from different selection rounds. All the displacement experiments were performed under the same conditions with various concentrations of picrotoxin or of RNA pools from different rounds. [^{3}H]TBOB (specific activity of 20 Ci/mmol, Amersham, final concentration of 20 nM), the membrane preparation (final concentration of 8−12 mg of fresh rat forebrain tissue/mL), and various concentrations of the displacing ligand, picrotoxin or RNAs from different selection rounds, were incubated in 400 μL of HEK external buffer at 37 °C for 2 h at pH 7.4. The concentration of the RNAs from each round was determined by measuring the absorbance at 260 nm (*49*). The reaction was terminated by filtering the mixture through Whatman GF/F filters on a 10-sample manifold (Hoefer FH225). The filters were then washed twice with 2 mL of ice-cold HEK 293 external buffer. The radioactivity retained on the filters was determined by liquid scintillation counting. (A) Displacement of 20 nM [^{3}H]TBOB bound to membrane fragments containing the $GABA_A$ receptor by picrotoxin. Approximately 60% of the total bound [^{3}H]TBOB was displaced by 100 μM picrotoxin. This value was taken to be 100% specific binding. The data are plotted according to eq III in the Appendix. Approximately 40% of total binding was subtracted in calculating the IC_{50} value. An IC_{50} value of 0.93 ± 0.33 μM for picrotoxin was obtained. (B) Displacement of 20 nM [^{3}H]TBOB bound to membrane fragments containing the $GABA_A$ receptor by RNAs in different selected rounds (200 nM) or by picrotoxin (≥100 μM). The control shows the amount of [^{3}H]TBOB bound to the membrane preparation. This is followed by the binding of round 0 (original RNA pool), round 5, round 11, and round 12, and of picrotoxin. The amount of [^{3}H]TBOB displaced from the membrane preparation by round 11 and round 12 of the RNA pool is approximately the same as the amount displaced by 100 μM picrotoxin. (C) Displacement of 20 nM [^{3}H]TBOB by the round 11 RNA pool. Unspecific binding was defined as the amount of [^{3}H]TBOB bound in the presence of 100 μM picrotoxin (Figure 2A). From the curve, the IC_{50} of round 11 RNA pool was determined to be 21.6 ± 6.4 nM, according to eq III in the Appendix.

RESULTS AND DISCUSSION

For the selection procedure, we used picrotoxin to displace the RNA ligands from the picrotoxin-binding site on the $GABA_A$ receptor in membrane fragments prepared from the rat forebrain. Because of the low concentration of picrotoxin-binding sites in the membrane and their instability, the quality of the membrane enriched in picrotoxin-binding sites is a key to ensuring selection success. Before using the membrane-bound picrotoxin site as the selecting target, the affinity of the membrane preparation for [^{3}H]TBOB (which binds to the same site as picrotoxin) (*29*, *39*, *40*) was determined. The displacement of [^{3}H]TBOB from the membrane preparation containing the $GABA_A$ receptor by picrotoxin is shown in Figure 2. Only ∼60% of 20 nM [^{3}H]TBOB was displaced by a saturating concentration of picrotoxin (Figure 2A). The amount not displaced is considered to be due to the [^{3}H]-TBOB nonspecifically bound to the membrane preparation (*33*, *35*). We have measured the level of nonspecific binding in the presence of up to 400 μM picrotoxin (Figure 2A). The displacement of [^{3}H]TBOB by a concentration of 200 nM round 0 and rounds 5, 11, and 12 of the increasingly enriched RNA pool (Figure 2B) indicates the differences in affinity of the different rounds for the TBOB (picrotoxin)-binding site. The ability of 200 nM round 11 or round 12 to displace [^{3}H]TBOB is approximately the same as that of 100 μM picrotoxin (Figure 2B). It is to be noticed (Figure 2B) that the specifically bound [^{3}H]TBOB is displaced to the same extent by 200 nM round 11 or 12 of the RNA pool. We also measured the displacement of 20 nM [^{3}H]TBOB by various concentrations of the enriched round 11 RNA pool (Figure 2C). The RNA pool of round 11 at various concentrations was incubated with 20 nM [^{3}H]TBOB and $GABA_A$ receptor-rich membrane fragments. The binding curve was fitted by eq III (see the Appendix). From the curve, the IC_{50} value of the round 11 pool was determined to be 21.6 ± 6.4 nM. The RNAs in the round 11 pool were cloned, and then sequenced by the Cornell University Biotechnology Resource Center. Fifty individual aptamers obtained from 96 clones were analyzed. These 50 aptamers were grouped into two classes based on their effects on the $GABA_A$ receptor in HEK 293 cells, as measured by the cell-flow technique (*17*, *19*, *37*, *38*). One group of 2′-F-RNA aptamers inhibited the $GABA_A$ receptor (for example, class I aptamer 95; see Figure 3); the second group did not inhibit the receptor. Among this second group, the aptamers shown in Table 1 alleviated picrotoxin inhibition of the $GABA_A$ receptor (Figure 4).

Sixteen of these aptamers had a definite consensus sequence, and they are listed in Table 1. They mainly fell into two broad categories, classes I and II. Eleven class I aptamers (Table 1) inhibit the $GABA_A$ receptor in the presence of 100 μM GABA in a concentration range of 2−60 nM. The results of a typical cell-flow experiment with a class I aptamer that inhibit the receptor are shown in Figure 3. This group has a UCUUGU consensus sequence. UCUUG occurs 100% of the time; the last nucleotide, U, occurs in eight of 11 class I aptamers. A GAAGCAAG consensus sequence also occurs frequently in this class. Twelve class II aptamers have the UUCACCGU consensus sequence. In the concentration range tested, up to 60 nM, five of these aptamers alleviate picrotoxin inhibition in the presence of

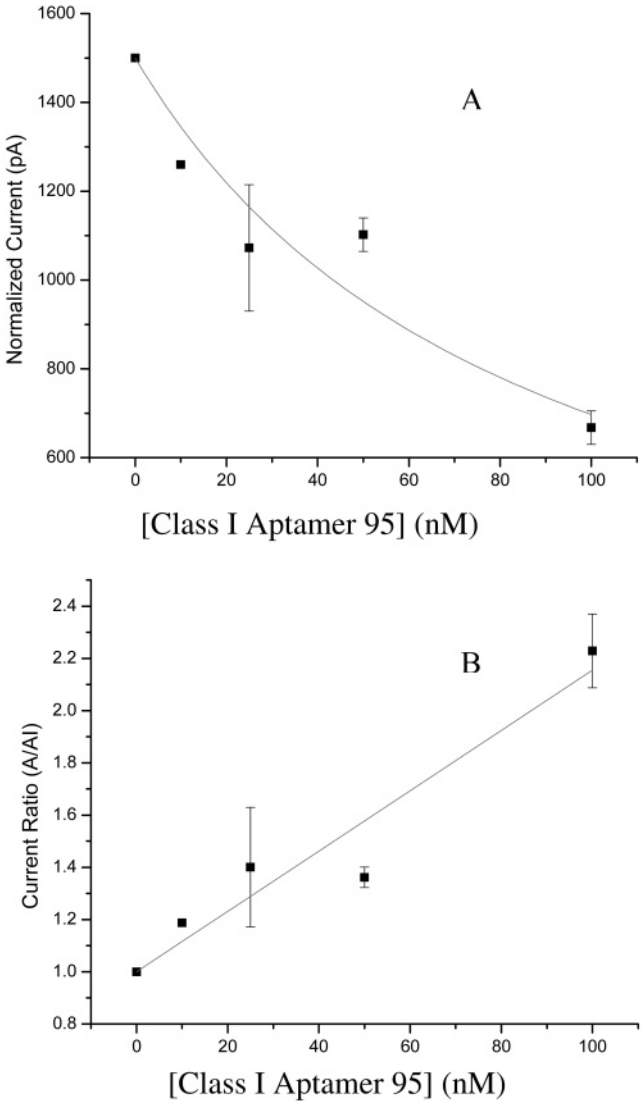

FIGURE 3: Inhibition of the $GABA_A$ receptor by class I aptamer 95. (A) The whole-cell current was recorded by using the cell-flow technique (*17*, *36*) at a constant concentration of 100 μM GABA and varying concentrations of class I aptamer 95 at a membrane potential of −60 mV and 22 °C in HEK 293 external buffer (pH 7.4). (B) Linear plot of the data in panel A. The data in panel A were plotted according to eq I in the Appendix. A is the corrected current in the presence of 100 μM GABA. A_I is the corrected current in the presence of 100 μM GABA and various concentrations of class I aptamer 95. From the slope of the line, the dissociation constant of the aptamer (K_{APT}) was determined to be 86 ± 8.6 nM.

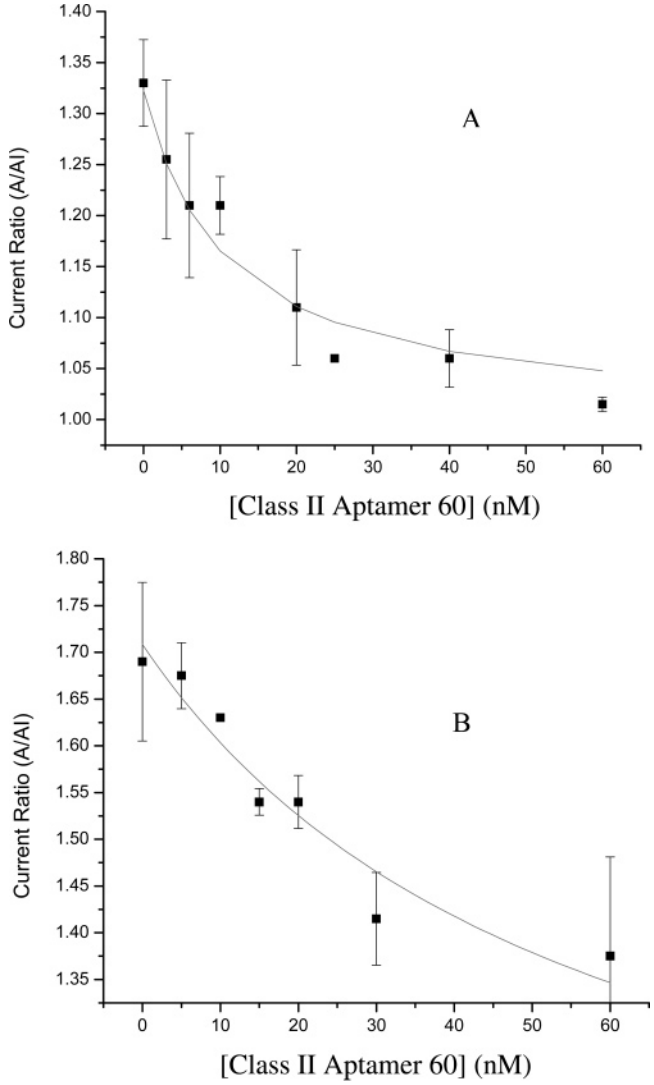

FIGURE 4: Alleviation of picrotoxin inhibition of the $GABA_A$ receptor by class II aptamer 60. The whole-cell currents were recorded (pH 7.4, 22 °C, and −60 mV) by using the cell-flow technique (*17*, *36*) and corrected for receptor desensitization (Experimental Procedures). GABA was the current-activating ligand, and 100 μM picrotoxin was the inhibitor. K_I(obs) and K_{APT}-(obs) represent the observed dissociation constants of the inhibitor (picrotoxin) and the aptamers, respectively. (A) Alleviation of inhibition of the $GABA_A$ receptor by 100 μM picrotoxin at a high concentration (100 μM) of GABA by class II 2′-F-RNA aptamer 60. The data were plotted according to eq II in the Appendix. K_I (for picrotoxin) = 310 ± 0.2 μM. K_{APT} (for the aptamer) = 10.5 ± 1.5 nM. (B) Alleviation of inhibition by 100 μM picrotoxin of the $GABA_A$ receptor at a low concentration of GABA (20 μM) by class II 2′-F-RNA aptamer 60. The data were plotted according to eq II in the Appendix. K_I (for picrotoxin) = 28 ± 0.9 μM. K_{APT} (for the aptamer) = 57.5 ± 9.5 nM.

100 μM GABA (Table 1). The other seven do not affect the $GABA_A$ receptor-mediated reaction. The results of a typical cell-flow experiments with a class II aptamer that alleviates picrotoxin inhibition of the $GABA_A$ receptor are shown in Figure 4. It may be of interest to look at the secondary structures of the aptamers that inhibit and alleviate $GABA_A$ receptor inhibition by picrotoxin (Figure 5). The different possible secondary structures, based on the sequences given in Table 1, were obtained by using MFOLD (http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi).

We obtained 12 additional aptamers for which we found no consensus sequence; some inhibited the receptor, and others alleviated receptor inhibition or had no effect on receptor function. This is in contrast to aptamers selected for the cocaine-binding site of the nAChR (*23*); aptamers that inhibited that receptor all had one consensus sequence, and aptamers that did not inhibit or alleviate inhibition all had another consensus sequence that was different from the sequence of the inhibitory aptamers (*23*).

Of the class II aptamers that alleviate picrotoxin inhibition of the $GABA_A$ receptor, aptamer 60 was chosen for more extensive study. Aptamer 60 exhibits a strong ability to alleviate picrotoxin inhibition as determined by the cell-flow technique (Figure 4). To determine the mechanism of action, the effect of aptamer 60 on the $GABA_A$ receptor was studied in the presence of both high (100 μM) and low (20 μM)

Table 1: Consensus Sequences and Affinities of Selected 2′-F-RNA Class I (UCUUGU) and Class II (UUCACCGU) Aptamers[a]

	Random region sequences (5'-3')	Inhibition of [^{3}H] TBOB binding by aptamers[b]
Class I 2'-F-RNA aptamers that inhibit the $GABA_A$ receptor		
Apt04	X-AGAGUCAUAGAG**UCUUGU**UAGUAACGGGAGCACCUAGGCA-Y	1.6
Apt08 (3)	X-CGAGCCGACGAGCGCAGAGUAGCAUAUGUGAAUU**UCUUGU**C-Y	1.8
Apt23 (5)	X-GAGAGGGAAGUAAGG**UCUUGU**ACCGGGCCUUCUGGACGUUA-Y	2.6
Apt47	X-GAGAGGUAAGUAAGG**UCUUGU**ACCGGGCCUUCUGGACGUUA-Y	1.5
Apt59	X-GGAGGGAAGUAAGA**UCUUGU**ACCCAUGACACGUCUACCGUG-Y	2.0
Apt95	X-GGAGGGAAGUAAGAU**UCUUGG**UGACAACGUCAUUAUACCAA-Y	2.3
Apt10	X-GAGGGGGAAGUAAGG**UCUUGU**ACCGGGCCUUCUGGACGA-Y	1.5
Apt38	X-GCGUGAAG**UCUUGG**GAGAGCAUUGGAUCGAACGUGUUGCGG-Y	1.7
Apt56	X-UCAUCAAGAUUCCUAA**UCUUGU**UGAGCGAUGGCCACCGACC-Y	2.2
Apt67	X-GAAAUGGAGUCUCUAGAAAUUGAGGACUAUAAGGU**UCUUGC**-Y	2.5
Apt87	X-GAGC**UCUUGU**UAAAGCAAGACCUCCAGAAGUGAGCGGGGA-Y	2.4
Consensus sequence	U C U U G U	
Frequency of nucleic acids in the sequence	11 11 11 11 11 8	
Class II 2'-F-RNA aptamers that alleviate picrotoxin inhibition of the $GABA_A$ receptor		
Apt9	X-GUGUCGGAAAAAGCAU**UUCACCGU**AGUCACCCAGAGGAGUC-Y	2.1
Apt19 (4)	X-CCGAGGAGG**UUCACCGU**AGUAUCCCUUGGGCCCACGCUGG-Y	2.3
Apt60 (2)	X-GGAGAGGGAGCGCUCAC**CUUCACCGU**GAACUCCCGGAGGC-Y	2.7
Apt73	X-CCGAGGAGG**UUCACCGU**AGUAUCCCUUGGGCCUACGCUGG-Y	1.8
Apt86	X-CCGAGGAGG**UUCACCGU**AGAAUCCCUUGGGCCAACGCUGG-Y	1.4
Consensus sequence	U U C A C C G U	
Frequency of nucleic acids in the sequence	5 5 5 5 5 5 5 5	

[a] The number in parentheses indicates the number of different colonies obtained in which the aptamer sequence is as shown. The underlined sequences show the consensus sequence: UCUUGU for class I and UUCACCGU for class II. X and Y represent flanking constant regions. The ability of the aptamers to inhibit or alleviate receptor inhibition by picrotoxin was assessed with HEK 293 cells expressing the $GABA_A$ receptor by using the cell-flow technique (*17*, *36*). The whole-cell current was recorded at pH 7.4, 22 °C, and −60 mV. GABA concentrations of 20 and 100 μM were used. In this concentration range, it can be estimated that the concentration of open receptor channels increases by a factor of ∼7 (*38*). [b] Displacement of bound [^{3}H]TBOB by aptamers is expressed as the ratio of the amount of [^{3}H]TBOB bound to $GABA_A$ receptor-rich membrane fragments (final concentration of 8−12 mg of fresh rat forebrain tissue/mL) in the absence and presence of 200 nM aptamers. [^{3}H]TBOB (20 nM) was used in these experiments.

concentrations of GABA. At high GABA concentrations, 70% of the $GABA_A$ receptor is in the open-channel form, whereas at low GABA concentrations, 10% of the receptor is in the open-channel form (*14*). When measurements are performed at a high concentration (100 μM) (Figure 4A) and then at a low concentration (20 μM) (Figure 4B) of GABA, the affinity of the receptor for picrotoxin increases. The value of K_I (the inhibitor dissociation constant of picrotoxin) decreases from 310 μM at high concentrations of GABA to 28 μM at low concentrations of GABA. In contrast, the affinity of the receptor for the aptamer decreases in going from high concentrations of GABA to low concentrations. The value of K_{APT} (the aptamer dissociation constant) increases from 10.5 nM at high concentrations of GABA (Figure 4A) to 57.5 nM at low concentrations of GABA (Figure 4B). Both results are in agreement with the proposed

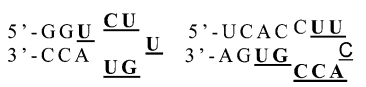

FIGURE 5: (A) Class I aptamer 23 (inhibitory) secondary structure. The UCUUGU consensus sequence formed a smaller stem−loop structure than the class II aptamer shown in panel B. (B) Class II aptamer 60 (alleviation) secondary structure. The UUCACCGU consensus sequence formed a stem−loop structure. Underlined nucleotides form the consensus sequence. The secondary structures of the aptamers were predicted using MFOLD (http://bioinfo.math-.rpi.edu/~infold/rna/form1.cgi).

mechanism of inhibition of the receptor by picrotoxin (*14*). The mechanism of inhibition of the $GABA_A$ receptor is similar to the mechanism of inhibition of the nAChR by cocaine and MK801 (Figure 1) (*15*, *41*, *42*). According to this mechanism, compounds that bind with higher affinity to a site on the closed-channel form of the receptor than to the same site on the open-channel form are expected to shift the channel-opening equilibrium toward the closed-channel form and inhibit the receptor. In contrast, compounds that bind to the same site as the noncompetitive inhibitor but with equal or higher affinity for the open-channel conformation will not change the channel-opening equilibrium (*15*). They are, therefore, not expected to inhibit the receptor but can displace the inhibitor (*15*).

Previous results (*38*) are consistent with the view that picrotoxin and [^{3}H]TBOB do not bind to the GABA-binding site of the $GABA_A$ receptor containing the $\alpha1$, $\beta2$, and $\gamma2$ subunits expressed in HEK 293 cells. Picrotoxin displaces the aptamers described here from their sites on the $GABA_A$ receptor. The simplest assumption is that these aptamers bind to the picrotoxin-binding site of the $GABA_A$ receptor and not to the GABA-binding site.

Previously, we have shown (*23*) that the SELEX technique (*24*, *25*) can be employed using membranes exceptionally rich in receptor sites, such as the nAChR in the electric organ of *Torpedo californica* (*23*). Here, we demonstrate that this technique can be used with a membrane preparation containing the relatively low concentration of a specific receptor in brain cells. The 2′-fluoro-modified RNA aptamers have a longer life in biological fluids or *in vivo* than the unmodified aptamers because of their stability (*26*, *43*, *44*) and are, therefore, expected to be particularly useful in investigations of the mechanism of the $GABA_A$ receptor in cells and living tissue. It has been shown that at least in one form of epilepsy the dysfunction of the $GABA_A$ receptor is due to an unfavorable channel-opening equilibrium (*8*). The use of the aptamers that alleviate inhibition of the $GABA_A$ receptor by picrotoxin, which changes the channel-opening equilibrium unfavorably (*14*), may be useful in developing drugs that alleviate some forms of epilepsy in which the channel-opening equilibrium is unfavorably affected.

APPENDIX

Analysis of Cell-Flow and Binding Data. In the case of an inhibitory aptamer (*17*, *45*):

$$A/A_I = 1 + [\text{Apt}]/K_{APT} \quad \text{(I)}$$

where A is the current amplitude of the control, A_I is the current amplitude in the presence of a selected RNA aptamer (Apt), and K_{APT} is the apparent dissociation constant of an inhibitory aptamer.

When various concentrations of an aptamer are used together with a constant concentration of inhibitor I (*17*, *45*):

$$A/A_I = 1 + [K_{APT}/(K_{APT} + [\text{Apt}])][I]/K_I \quad \text{(II)}$$

where A_I is the current amplitude in the presence of both the aptamer and a noncompetitive inhibitor such as picrotoxin and K_{APT} and K_I are the observed dissociation constants of the aptamer and the inhibitor (picrotoxin), respectively.

According to eq II, the slope of the ratio of the current amplitudes in the absence and presence of both the aptamer and inhibitor as a function of inhibitor concentration, but in the presence of a fixed concentration of the aptamer, will decrease by a factor of $K_{APT}/(K_{APT} + [\text{Apt}])$ compared with the slope for the inhibitor alone (slope $= 1/K_I$). This relationship is based on the assumption that the selected 2′-F-RNA aptamer binds to the same site as does the inhibitor, thus preventing picrotoxin from binding to it.

Binding data curve fitting (*46*):

$$Y = B_{min} + (B_{max} - B_{min})/(1 + [\text{inhibitor}]/IC_{50}) \quad \text{(III)}$$

where Y is the total binding of [^{3}H]TBOB in the presence of various concentrations of the inhibitor, such as picrotoxin, or an aptamer, B_{max} is the maximum total amount of [^{3}H]-TBOB bound, and B_{min} is the amount of [^{3}H]TBOB non-specifically bound to the $GABA_A$ receptor that cannot be displaced by an excess of ligand competing for the picrotoxin-binding site. IC_{50} is the concentration at which a compound displaces 50% of [^{3}H]TBOB from the membrane.

ACKNOWLEDGMENT

We thank Susan Coombs for editorial help in preparing the manuscript and Dr. R. F. Squires for helpful discussions concerning the preparation of the rat forebrain membrane.

REFERENCES

1. Kandel, E. R., Schwartz, J. H., and Jessel, T. M. (1995) *Essentials of Neural Science and Behavior*, Apleton & Lange, Stamford, CT.
2. Bormann, J., and Kettenmann, H. (1988) Patch-clamp study of γ-aminobutyric acid receptor Cl^- channels in cultured astrocytes, *Proc. Natl. Acad. Sci. U.S.A. 85*, 9336−9340.
3. Mohler, H. (1992) GABAergic synaptic transmission. Regulation by drugs, *Arzneimittelforschung 42*, 211−214.
4. Olsen, R. W., and Avoli, M. (1997) GABA and epileptogenesis, *Epilepsia 38*, 399−407.
5. Olsen, R. W., DeLorey, T. M., Gordey, M., and Kang, M. H. (1999) GABA receptor function and epilepsy, *Adv. Neurol. 79*, 499−510.
6. Jayaraman, V., and Thiran, S. (2000) A mechanistic investigation of the function of γ-aminobutyric acid (λ) receptor in a genetically epileptic prone animal model, Meeting Abstract, *Biophys. J. 78*, 2083a.
7. Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuern, E. (2001) First genetic evidence of GABA(A) receptor dysfunction in epilepsy: A mutation in the γ2-subunit gene, *Nat. Genet. 28*, 46−48.
8. Ramakrishnan, L., and Hess, G. P. (2004) On the mechanism of a mutated and abnormally functioning γ-aminobutyric acid A receptor linked to epilepsy, *Biochemistry 43*, 7534−7540.
9. Sieghart, W. (1995) Structure and pharmacology of γ-aminobutyric acid A receptor subtypes, *Pharmacol. Rev. 47*, 181−234.
10. De Deyn, P. P., Marescau, B., and MacDonald, R. L. (1990) Epilepsy and the GABA hypothesis: A brief review and some examples, *Acta Neurol. Belg. 90*, 65−81.

11. Squires, R. F., Casida, J. E., Richardson, M., and Saederup, E. (1983) [^{35}S]-*tert*-Butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric acid-A and ion recognition sites, *Mol. Pharmacol. 23*, 326−336.
12. Levine, J. A., Ferrendelli, J. A., and Covey, D. F. (1985) Convulsant and anti-convulsant γ-butyrolactones bind at the picrotoxinin/*tert*-butylbicyclophosphorothionate (TBPS) receptor, *Biochem. Pharmacol. 34*, 4187−4190.
13. Holland, K. D., McKeon, A. C., Covey, D. F., and Ferrendelli, J. A. (1990) Binding interactions of convulsant and anticonvulsant γ-butyrolactones and γ-thiobutyrolactones with the picrotoxin receptor, *J. Pharmacol. Exp. Ther. 254*, 578−583.
14. Ramakrishnan, L., and Hess, G. P. (2004) Picrotoxin inhibition mechanism of the γ-aminobutyric acid A receptor investigated by a laser-pulse photolysis technique, *Biochemistry* (submitted for publication).
15. Hess, G. P., Ulrich, H., Breitinger, H. G., Niu, L., Gameiro, A. M., Grewer, C., Srivastava, S., Ippolito, J. E., Lee, S. M., Jayaraman, V., and Coombs, S. E. (2000) Mechanism-based discovery of ligands that counteract inhibition of the nicotinic acetylcholine receptor by cocaine and MK-801, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13895−13900.
16. Neher, E., and Sakmann, B. (1976) Single-channel currents recorded from membrane of denervated frog muscle fibers, *Nature 260*, 799−802.
17. Udgaonkar, J. B., and Hess, G. P. (1987) Chemical kinetic measurements of a mammalian acetylcholine receptor by a fast-reaction technique, *Proc. Natl. Acad. Sci. U.S.A. 84*, 8758−8762.
18. Milburn, T., Matsubara, N., Billington, A. P., Udgaonkar, J. B., Walker, J. W., Carpenter, B. K., Webb, W. W., Marque, J., Denk, W., McCray, J. A., and Hess, G. P. (1989) Synthesis, photochemistry, and biological activity of a caged photolabile acetylcholine receptor ligand, *Biochemistry 28*, 49−55.
19. Matsubara, N., Billington, A. P., and Hess, G. P. (1992) How fast does an acetylcholine receptor channel open? Laser-pulse photolysis of an inactive precursor of carbamoylcholine in the microsecond time region with BC3H1 cells, *Biochemistry 31*, 5507−5514.
20. Hess, G. P., Cash, D. J., and Aoshima, H. (1979) Acetylcholine receptor-controlled ion fluxes in membrane vesicles investigated by fast reaction techniques, *Nature 282*, 329−331.
21. Sakmann, B., Patlak, J., and Neher, E. (1980) Single acetylcholine-activated channels show burst-kinetics in the presence of desensitizing concentrations of agonist, *Nature 286*, 71−73.
22. Hammes, G. (2000) *Thermodynamics and Kinetics for the Biological Sciences*, Wiley, New York.
23. Ulrich, H., Ippolito, J. E., Pagan, O. R., Eterovic, V. A., Hann, R. M., Shi, H., Lis, J. T., Eldefrawi, M. E., and Hess, G. P. (1998) *In vitro* selection of RNA molecules that displace cocaine from the membrane-bound nicotinic acetylcholine receptor, *Proc. Natl. Acad. Sci. U.S.A. 95*, 14051−14056.
24. Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage-T4 DNA-polymerase, *Science 249*, 505−510.
25. Ellington, A. D., and Szostak, J. W. (1990) In vitro selection of RNA molecules that bind specific ligands, *Nature 346*, 818−822.
26. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., and Eckstein, F. (1991) Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes, *Science 253*, 314−317.
27. Lin, Y., Qiu, Q., Gill, S. C., and Jayasena, S. D. (1994) Modified RNA sequence pools for in vitro selection, *Nucleic Acids Res. 22*, 5229−5234.
28. Aurup, H., Williams, D. M., and Eckstein, F. (1992) 2′-Fluoro-2′-deoxynucleoside and 2′-amino-2′-deoxynucleoside 5′-triphosphates as substrates for T7 RNA-polymerase, *Biochemistry 31*, 9636−9641.
29. Squires, R. F., and Saederup, E. (2000) Additivities of compounds that increase the numbers of high affinity [^{3}H]muscimol binding sites by different amounts define more than 9 GABA(A) receptor complexes in rat forebrain: Implications for schizophrenia and clozapine research, *Neurochem. Res. 25*, 1587−1601.
30. Squires, R. F., Ai, J., Witt, M. R., Kahnberg, P., Saederup, E., Sterner, O., and Nielsen, M. (1999) Honokiol and magnolol increase the number of [^{3}H]muscimol binding sites three-fold in rat forebrain membranes in vitro using a filtration assay, by allosterically increasing the affinities of low-affinity sites, *Neurochem. Res. 24*, 1593−1602.
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
32. Fitzwater, T., and Polisky, B. (1996) A SELEX primer, *Methods Enzymol. 267*, 275−301.
33. Lawrence, L. J., Palmer, C. J., Gee, K. W., Wang, X., Yamamura, H. I., and Casida, J. E. (1985) *tert*-[^{3}H]Butylbicycloorthobenzoate: New radioligand probe for the γ-aminobutyric acid-regulated chloride ionophore, *J. Neurochem. 45*, 798−804.
34. Vanrijn, C. M., Willemsvanbree, E., Vandervelden, T. J. A. M., and Demiranda, J. F. R. (1990) Binding of the cage convulsant, [^{3}H]TBOB, to sites linked to the GABA-A receptor complex, *Eur. J. Pharmacol. 179*, 419−425.
35. Vanrijn, C. M., Vanbree, E. W., Dirksen, R., and Demiranda, J. F. R. (1992) The GABA-A receptor complex in relation to epilepsy: reversal of [^{3}H]TBOB inhibition. A prediction of proconvulsive properties, *Epilepsy Res. 12*, 163−170.
36. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pfluegers Arch. 391*, 85−100.
37. Hess, G. P. (1993) Determination of the chemical mechanism of neurotransmitter receptor-mediated reactions by rapid chemical kinetic techniques, *Biochemistry 32*, 989−1000.
38. Jayaraman, V., Thiran, S., and Hess, G. P. (1999) How fast does the γ-aminobutyric acid receptor channel open? Kinetic investigations in the microsecond time region using a laser-pulse photolysis technique, *Biochemistry 38*, 11372−11378.
39. Squires, R. F., and Saederup, E. (1987) $GABA_A$ receptor blockers reverse the inhibitory effect of GABA on brain-specific [^{35}S]TBPS binding, *Brain Res. 414*, 357−364.
40. Pericic, D., Mirkovic, K., Jazvinscak, M., and Besnard, F. (1998) [^{3}H]-*tert*-Butylbicycloorthobenzoate binding to recombinant $\alpha1\beta2\gamma2$s GABA(A) receptor, *Eur. J. Pharmacol. 360*, 99−104.
41. Grewer, C., and Hess, G. P. (1999) On the mechanism of inhibition of the nicotinic acetylcholine receptor by the anticonvulsant MK-801 investigated by laser-pulse photolysis in the microsecond-to-millisecond time region, *Biochemistry 38*, 7837−7846.
42. Hess, G. P., Gameiro, A. M., Schoenfeld, R. C., Chen, Y., Ulrich, H., Nye, J. A., Sit, B., Carroll, F. I., and Ganem, B. (2003) Reversing the action of noncompetitive inhibitors (MK-801 and cocaine) on a protein (nicotinic acetylcholine receptor)-mediated reaction, *Biochemistry 42*, 6106−6114.
43. Pagratis, N. C., Bell, C., Chang, Y. F., Jennings, S., Fitzwater, T., Jellinek, D., and Dang, C. (1997) Potent 2′-amino- and 2′-fluoro-2′-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor, *Nat. Biotechnol. 15*, 68−73.
44. Cui, Y., Ulrich, H., and Hess, G. P. (2004) Selection of 2′-fluoro-modified RNA aptamers for alleviation of cocaine and MK-801 inhibition of the nicotinic acetylcholine receptor, *J. Membr. Biol.* (in press).
45. Hess, G. P., and Grewer, C. (1998) Development and application of caged ligands for neurotransmitter receptors in transient kinetic and neuronal circuit mapping studies, *Methods Enzymol. 291*, 443−473.
46. McPherson, G. A. (1985) Analysis of radioligand binding experiments: A collection of computer-programs for the IBM-PC, *J. Pharmacol. Methods 14*, 213−228.
47. Katz, B., and Thesleff, S. (1957) A study of the desensitization produced by acetylcholine at the motor end-plate, *J. Physiol. 138*, 63−80.
48. Cash, D. J., Aoshima, H., and Hess, G. P. (1981) Acetylcholine-induced cation translocation across cell membranes and inactivation of the acetylcholine receptor: Chemical kinetic measurements in the millisecond time region, *Proc. Natl. Acad. Sci. U.S.A. 78*, 3318−3322.
49. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.

BI048667B