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ORIGINAL ARTICLES

Minimal RNA Aptamer Sequences That Can Inhibit or Alleviate Noncompetitive Inhibition of the Muscle-Type Nicotinic Acetylcholine Receptor

Kannan Sivaprakasam · Oné R. Pagán · George P. Hess

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Abstract Combinatorially synthesized nucleotide polymers have been used during the last decade to find ligands that bind to specific sites on biological molecules, including membrane-bound proteins such as the nicotinic acetylcholine receptors (nAChRs). The neurotransmitter receptors belong to a group of four structurally related proteins that regulate signal transmission between $\sim 10^{11}$ neurons of the mammalian nervous system. The nAChRs are inhibited by compounds such as the anticonvulsant MK-801 [(+)-dizocilpine] and abused drugs such as cocaine. Based on predictions arising from the mechanism of receptor inhibition by MK-801 and cocaine, we developed two classes of RNA aptamers: class I members, which inhibit the nAChR, and class II members, which alleviate inhibition of the receptor by MK-801 and cocaine. The systematic evolution of ligands by the exponential enrichment (SELEX) method was used to obtain these compounds. Here, we report that we have truncated RNA aptamers in each class to determine the minimal nucleic acid sequence that retains the characteristic function for which the aptamer was originally selected. We demonstrate that a truncated class I aptamer containing a sequence of seven nucleotides inhibits the nAChR and that a truncated class II aptamer containing a sequence of only four nucleotides can alleviate MK-801 inhibition.

Keywords Nicotinic acetylcholine receptor · Ribonucleic acid · Deoxyribonucleic acid · Systematic evolution of ligands by exponential enrichment (SELEX) · Cocaine · Aptamer · Phencyclidine · MK-801 (+)-dizocilpine · Whole-cell current recording

Introduction

The nicotinic acetylcholine receptors (nAChRs) are transmembrane proteins formed by pentameric arrangements of subunits. They are a widely studied prototype of ligand-gated ion channels (Karlin 2002; Le Novere et al. 2002; Unwin 2005). They mediate neurotransmission at the neuromuscular junction, in the autonomic ganglia and in the central nervous system (Schapira et al. 2002). When activated by acetylcholine, the receptors transiently (~ 1 ms) form transmembrane channels, allowing cations to cross the cell membrane (reviewed in Karlin 2002). This changes the transmembrane voltage of the cell membrane and initiates signal transmission between cells in the nervous system. The receptors are the target of noncompetitive inhibitors (Fig. 1b), such as the local anesthetics procaine and QX-222, MK-801 [(+)-dizocilpine], which has anticonvulsant properties, and abused drugs, including phencyclidine (PCP) and cocaine (Aguayo and Albuquerque

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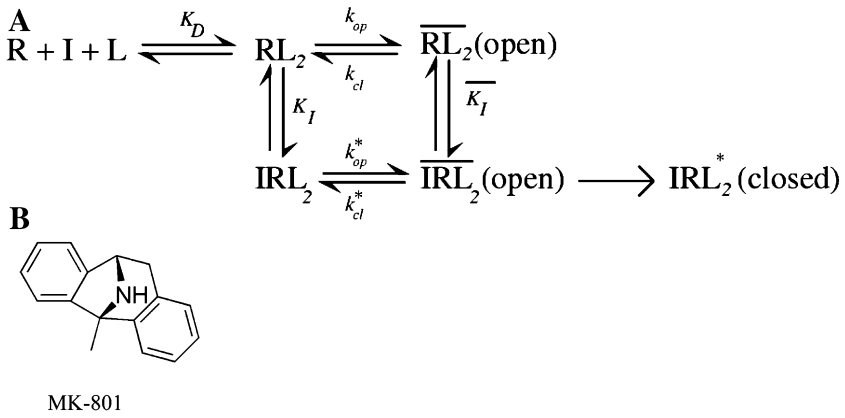

Fig. 1 **a** The mechanism shown (Hess et al. 2000) accounts for the alleviation of inhibition of the nAChR by cocaine and MK-801 by ligands that displace these noncompetitive inhibitors from their binding site without changing the channel-opening equilibrium unfavorably and, therefore, without affecting the receptor function. K_D is the apparent dissociation constant for the activating ligand L, K_I the apparent dissociation constant for the inhibitor from the closed-channel forms of the receptor (RL and RL_2) and $\overline{K_I}$the apparent dissociation constant of the inhibitor bound to the open-channel forms of the receptor $\overline{RL_2}$ and $\overline{IRL_2}$, respectively. *Subscript 2* denotes the number of ligand molecules bound to the receptor. Φ^{-1} is the equilibrium constant for the conversion of RL_2 to $\overline{RL_2}$ ($\Phi^{-1} = \overline{RL_2}/RL_2$), and Φ_I^{-1} is the equilibrium constant for the conversion of IRL_2 to $\overline{IRL_2}$ (open). The principle of microscopic reversibility (Hammes 2000) requires that $\Phi_I^{-1}/\Phi^{-1} = K_I/\overline{K_I}$. This relationship indicates that (1) ligands that bind to a regulatory site on the RL_2 form with higher affinity than to the site on the open-channel form $\overline{RL_2}$(*open*) will shift the equilibrium from the open-channel conformation toward the closed-channel conformation and inhibit the receptor (Hess et al. 2000)—class I RNA aptamers with the consensus sequence UUCACCG belong to this group—and (2) ligands that bind with equal or higher affinity to the open-channel conformation than to the closed-channel conformation will not change the channel-opening equilibrium in an unfavorable way (Hess et al. 2000)—these ligands will not, therefore, inhibit the receptor-mediated reaction but can prevent the binding and action of inhibitors that bind to the same sites but with higher affinity to the RL_2 than the $\overline{RL_2}$ form; class II RNA aptamers exhibit these properties. **b** Structure of MK-801, a noncompetitive inhibitor of the nAChR

1986; Ramoa et al. 1990; Niu and Hess 1993; Niu et al. 1995; Hardman et al. 1996; Katz et al. 1997).

The single-channel current-recording technique (Sakmann and Neher 1995; Colquhoun and Sakmann 1998) has been supplemented by transient kinetic techniques suitable for measurements of receptor-mediated reactions on cell surfaces in the microsecond to millisecond time region (Krishtal and Pidoplichko 1980; Udgaonkar and Hess 1987; Milburn et al. 1989; Matsubara et al. 1992; Niu and Hess 1993; Hess and Grewer 1998; Hess 2003, 2005). The laser-pulse photolysis (LaPP) technique (reviewed in Hess and Grewer 1998; Hess 2003, 2005) allows one to determine the effects of drugs that act on the receptor. By using this technique, the rate constants for both channel opening (k_{op}) and closing (k_{cl}) of the receptor (Fig. 1a) and, therefore, the channel-opening equilibrium constant ($\Phi^{-1} = k_{op}/k_{cl}$) can be determined, all in the same experiment (Milburn et al. 1989; Matsubara et al. 1992; Hess and Grewer 1998; Hess 2003). The results of LaPP experiments indicated that in noncompetitive inhibition of the nAChR by cocaine, PCP and MK-801 [(+)-dizocilpine] these compounds bind with higher affinity to the closed-channel form than to the open-channel form of the receptor, resulting in an inhibitor-induced decrease in the channel-opening equilibrium constant (Fig. 1a) (Grewer and Hess 1999). This suggested that compounds might be found that bind to an inhibitory site on the open-channel receptor form with equal or higher affinity than to the inhibitory site on the closed-channel receptor form. Thus, they are not expected to adversely affect the channel-opening equilibrium constant (Fig. 1a) (Grewer and Hess 1999) but can still displace noncompetitive inhibitors, such as MK-801 and cocaine, thus alleviating their inhibition of the receptor. We demonstrated that combinatorially synthesized RNA ligands (aptamers) with these properties can be isolated by displacing with cocaine aptamers that were bound with high affinity to the nAChR (Ulrich et al. 1998; Hess et al. 2000).

The systematic evolution of ligands by the exponential enrichment (SELEX) method has been used for the isolation of DNA/RNA molecules from large pools (10^{24}–10^{50} molecules) of combinatorially synthesized nucleic acid molecules that bind to a wide range of targets with high specificity and affinity (Tuerk and Gold 1990; Ellington and Szostak 1990). The targets have included ribonucleases (Hirao et al. 2004), bovine thrombin (Liu et al. 2004) and carcinogenic aromatic amines (Brockstedt et al. 2004), among others (Blank and Blind 2005). The SELEX method can also be used for the development of clinically useful therapeutic agents (Gold 1995; Jayasena 1999; Pendergrast et al. 2005; Proske et al. 2005; Ng et al. 2006). The isolation of RNA aptamers that compete with MK-801, PCP

or cocaine (Fig. 1b) for a binding site on the nAChR was accomplished (Ulrich et al. 1998) using the electroplax membrane of *Torpedo californica*, which is rich in the receptor. RNA aptamers isolated for the cocaine-binding site are also effective against MK-801 inhibition of the nAChR (Hess et al. 2000). Since MK-801 and cocaine share one of the binding sites on the nAChR (Ulrich et al. 1998) and inhibit the receptor by the same mechanism (Niu and Hess 1993; Niu et al. 1995), we used MK-801 throughout the experiments described here.

The mechanism, proposed on the basis of transient kinetic measurements (reviewed in Hess and Grewer 1998), predicted the existence of compounds that can bind to a site on the nAChR to which noncompetitive inhibitors also bind but, in contrast to the inhibitors, without changing the channel-opening equilibrium constant (Hess et al. 2000). The mechanism-based (see Fig. 1a) approach also led to the first identification of small organic molecules that can alleviate MK-801 and cocaine inhibition of the nAChR (Hess et al. 2003; Chen et al. 2004). These compounds bind to the noncompetitive inhibitory site on the nAChR but, unlike inhibitors, bind to the open-channel form of the receptor with equal or higher affinity than to the closed-channel conformation. An example of such a compound is ecgonine methyl ester (Chen et al. 2004). Therefore, they do not inhibit the receptor but can counteract its inhibition by compounds such as cocaine that bind with higher affinity to the closed- than the open-channel form (Ulrich et al. 1998; Hess et al. 2000, 2003).

The combinatorially synthesized RNA ligands contain 90 nucleotides (nt) (Fig. 2a; see supplementary information, Tables S1 and S2, and Ulrich et al. 1998 [supplementary information obtained by Ulrich et al. 1998, is available for this article at http://dx.doi.org/.]) and are much larger than the inhibitors they displace from the nAChR. Here, we identify the minimum size of the

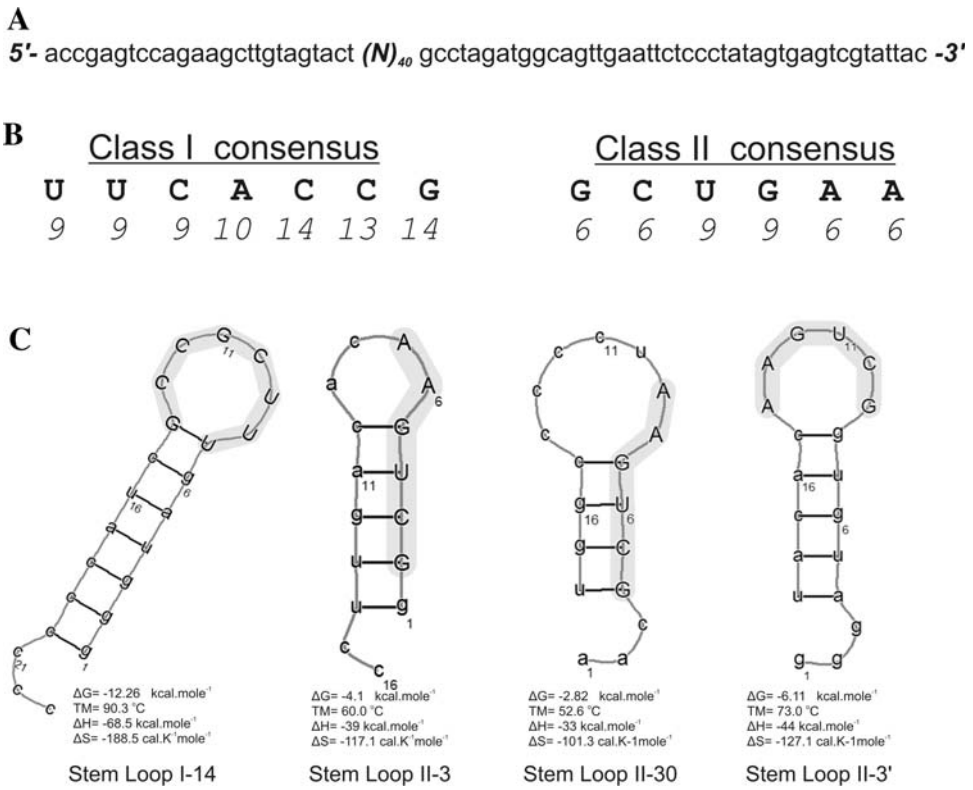

Fig. 2 The RNA aptamers isolated by the SELEX technique fall into two classes based on their function, class I aptamers inhibit the nAChR and class II aptamers alleviate noncompetitive inhibition of the receptor. Upon sequencing (Cornell Biotechnology Resource Center), the two classes were found to have characteristic consensus sequences (Ulrich et al. 1998). **a** The DNA template used in the selection process consisted of 108 nt comprising two constant regions flanking a variable sequence of 40 nt, $(N)_{40}$ (Ulrich et al. 1998). **b** The consensus sequences of the two classes of RNA aptamers are written from 5′ to 3′. Fourteen class I aptamers and ten class II aptamers were obtained. The frequency of occurrence of each of the nucleotides within the consensus sequence is given below the bases. **c** The sequences of the truncated stem loops of aptamers I-14, II-3, II-30 and II-3′ are given in the 5′–3′ direction. (The prime is used to distinguish stem loop II-3′ from the stem loop II-3, with which it shares the consensus sequence.) Consensus sequences are highlighted. Zuker's mfold program was used to predict the most stable secondary structure of the RNA aptamers (Zuker 2003). It can be accessed online at http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi. The estimated melting temperature (T_M) and thermodynamic parameters ΔG, ΔH and ΔS are given below each structure

nucleotide sequence responsible for the biological effect of the aptamer. Analysis of the sequences of the 24 RNA aptamers obtained previously (Ulrich et al. 1998) indicated that on a functional and sequence basis they fall into two distinct classes. Members of class I bind with higher affinity to the closed-channel form of the nAChR than to the open-channel form and inhibit the receptor. Members of class II bind with equal affinity to the closed- and open-channel forms; thus, they do not inhibit the receptor but are able to prevent the binding of noncompetitive inhibitors that bind with higher affinity to the closed-channel form (Hess et al. 2000). Each class of aptamer is characterized by a different consensus sequence of seven (class I) or six (class II) nucleotides (Fig. 2b).

It is widely established that conserved sequence motifs (consensus sequences) play an important role in biological functions (e.g., Szostak 1986; Tuerk and Gold 1990; Park and Sternglanz 1998; Eder et al. 2000; Ansai et al. 2002; Robertson et al. 2005). Our strategy to determine minimal functional sequences within the isolated aptamers involved gradually truncating aptamers but keeping the consensus sequence intact. We first studied the biological activities of reduced chain lengths containing the consensus sequence of the most potent molecules in each of the two classes of aptamers. We then determined whether nucleotide polymers consisting of consensus sequences (Fig. 2b) inhibit the receptor (class I) or alleviate receptor inhibition (class II). As a final step, we determined the minimum number of nucleotides within the consensus sequence responsible for biological activity. Four nucleotides of the class II consensus sequence of six nucleotides are required for the alleviation of MK-801 inhibition of the muscle-type nAChR (Table 1). In the case of the aptamers that inhibit the nAChR, all seven nucleotides of the class I consensus sequence are required (Table 1).

Materials and Methods

Materials

All chemicals used were of the highest quality available. Carbamoylcholine and MK-801 [(+)-dizocilpine)] were purchased from Sigma (St. Louis, MO). The chemicals for cell culture and buffers were purchased from Invitrogen BRL (Grand Island, NY) and Sigma. T7 RNA polymerase,

Table 1 Aptamers tested and their apparent dissociation constants as measured in electrophysiological assays

	Number of nucleotides	Molecular weight (daltons)[a]	K_{Iapt} or K_{Apt} (μM)
Class I aptamers			K_{Iapt}
I-14 Full-length (see sequence in supplementary information, Table S1)	90	29,096	0.5 [b]
I-14 Stem loop gggUAGU**UUC**G**CCGC**UAcccccc	20	7.471	37 ± 3
Class I consensus gg**UUCACCG**	10	3,052	33 ± 4.8
UUCACCG	7	2,361	58 ± 4
ggTTCACCG[b]	9	2,715	74 ± 3
Class II aptamers			K_{Apt}
II-3 Full-length (see sequence in supplementary information, Table S2)	90	29,112	0.3 ± 0.15 [b]
II-3 Stem loop G**GCUGAA**CACAGUUcc	18	5,301	3 ± 1.4
II-3	7	2,464	4 ± 1.5
g**GCUGAA**			
gg**GCUG**	6	2,151	55 ± 5
II-3' Stem loop gggAUGUG**GCUGAA**CACAU	19	6,362	–
II-30 Full-length (see sequence in supplementary information, Table S2)	89	28,782	0.8 ± 0.45
II-30 Stem loop AAC**GCUGAA**UCCCCCGGU)	21	5,911	16 ± 8
gggGCTGAA	9	2,804	15 ± 5

[a] Molecular weights determined using http://www.basic.northwestern.edu/biotools/oligocalc.html

[b] The values of K_{Iapt} for aptamer I-14 and K_{Apt} for II-3 aptamer were determined previously (Ulrich et al. 1998; Hess et al. 2000)

K_{Apt} is the apparent dissociation constant of the aptamers that alleviate receptor inhibition, and K_{Iapt} is the apparent dissociation constant of the aptamers that inhibit the receptor. The apparent dissociation constants were determined from measurements of the whole-cell current and use of the cell-flow method with BC_3H1 cells containing the fetal muscle-type nAChR (Figs. 3, 4, and 5). Their values were computed from the measurements by using Eqs. 1 and 2 (Hess and Grewer 1998) (see "Materials and Methods"). The measurements were made at approximately 22°C, pH 7.4 and –60 mV in bath buffer (the composition is given in "Materials and Methods"). The sequences are written in the 5′–3′ direction. The consensus sequence is indicated by underlined uppercase bold letters

anti-ribonuclease (Superase) and deoxyribonuclease (DNase) were obtained from Ambion (Austin, TX). The ribonucleotides were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Standard precautions were taken to prevent ribonuclease (RNase) contamination of Eppendorf tubes. Glassware was autoclaved at 180°C, and buffers were sterilized by passing them through cellulose acetate filters with 2-μm diameter pores (Nalgene Labware, Rochester, NY). The sources of instruments and analysis programs used are noted in the individual sections.

Synthesis and Purification of RNA Aptamers

Full-length class I-14, class II-3 and II-30 aptamers were prepared as described previously (Ulrich et al. 1998). Most of the truncated RNA aptamers discussed here were enzymatically synthesized from a DNA template (Milligan and Uhlenbeck 1989). The DNA templates were obtained by annealing chemically synthesized DNA sequences containing the T7 promoter primer (5′- TAATACGA CTCACTATAGGG-3′), which is constant for all the aptamers used, with the nucleotide strand containing the variable sequences and the flanking constant, nontranscribed sequence 5′-CCCTATAGTGAGTCGTATTAGG ATCC-3′. These oligonucleotides were synthesized using phosphoramidate chemistry (Kates and Albericio 2000) and purified by high-performance liquid chromatography (HPLC) by the Cornell Bioresource Center. In vitro transcription of a partially double-stranded DNA template (only the sequence containing the T7 promoter primer was double-stranded) was carried out using T7 RNA polymerase at 37°C. An incubation period of 8–10 h was used. The residual DNA templates were digested by incubating them in the presence of RNase-free DNase 1 (2 U/100 μl transcription reaction, Ambion) at 37°C for 15 min. The purified RNA was obtained after phenol-chloroform washing and ethanol precipitation (Ausubel et al. 1999). In vitro transcribed RNAs contain guanosine residues at the 5′ end (Jia and Patel 1997). The truncated RNA molecules UUCACCG, CUCAGCG, UCACCG, GCUGAA, GCACCG, GCUG and UCGG were chemically synthesized and then purified by HPLC, all by Syngen (San Carlos, CA). DNA versions of the short RNA aptamers ggTTCACCG and gggGCTGAA were also chemically synthesized and then purified by HPLC, all by Integrated DNA Technologies (Coralville, IA). They consist of a minimum of nine nucleotides containing 5′ guanosine residues.

Cell Culture

The BC_3H1 cell line expressing the embryonic mouse muscle-type nAChR was cultured as described elsewhere (Schubert et al. 1974). BC_3H1 cells were grown in 25-cm^2 canted-neck cell culture flasks (Corning Glass Works, Corning, NY) containing the growth medium Dulbecco's modified Eagle medium, high glucose (DMEM, Invitrogen BRL), supplemented with 10% fetal bovine serum (FBS, Invitrogen BRL) and antibiotics (100 IU of penicillin, 100 μg/L streptomycin; both from Sigma). Cells were passaged weekly (after reaching 80–90% confluence). For measurements, 35-mm Falcon dishes (Fisher Scientific, Pittsburgh, PA) were seeded with $\sim 10^5$ cells in 1% FBS and 99% DMEM containing 1% penicillin/streptomycin (Invitrogen BRL).

Whole-Cell Current Recording

Current was recorded in the whole-cell configuration (Hamill et al. 1981). Typical pipette resistances were 2–4 MΩ and the series resistance was 1–4 MΩ for whole-cell current measurements. Series resistance compensation of 60–70% was used in the whole-cell current-recording experiments. The solution in the recording pipette contained (in mM) 140 potassium chloride, 10 sodium chloride, 2 magnesium chloride, 1 ethylene glycol-bis-(2-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EDTA) and 25 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), adjusted to pH 7.4. The extracellular buffer contained (in mM) 145 sodium chloride, 5.3 potassium chloride, 1.8 calcium chloride, 1.2 magnesium chloride and 25 HEPES, adjusted to pH 7.4. The whole-cell currents were amplified by using an Axon amplifier 200B (Axon Instruments, Union City, CA). Signal acquisition was carried out by using a Labmaster DMA 100 kHz digitizing board (Scientific Solutions, Chelmsford, MA) controlled by pClamp 8 (Axon Instruments, Union City, CA) software. The current signal was low pass–filtered at 1 kHz using the amplifier's built-in circuitry (4-pole low-pass Bessel filter) and digitized at 200–800 Hz. Data were analyzed offline on a PC, and the time constants for the rising and decaying phases of the whole-cell current were obtained by using a nonlinear least-squares fitting program with Origin 3.5 software (OriginLab, Northampton, MA). All measurements were carried out at pH 7.4, 22°C and a transmembrane voltage of −60 m*V*. Each data point represents the average of three to six measurements made with at least two independent cells.

Cell-Flow Method

Equilibration of ligands with nAChRs on the BC_3H1 cell surface was achieved by use of the cell-flow method as described earlier (Udgaonkar and Hess 1987). The time resolution of this technique with the cells used is 10 ms. The observed current must be corrected for receptor

desensitization, which occurs while the neurotransmitter equilibrates with the cell surface receptors. The theory and procedure to make this correction have been described (Udgaonkar and Hess 1987). Cells were allowed to recover for 2 min after each measurement, a time sufficient to guarantee full resensitization of the receptors (Raines and Zachariah 1999). All RNA aptamer solutions contained 0.11 U/μl of a dithiothreitol-independent RNase inhibitor, anti-RNase (Superase, Ambion), a concentration that had no effect on receptor activity when tested in cell-flow experiments but that does ensure the stability of the RNA aptamer (Ulrich et al. 1998). The RNA aptamers were preincubated with the cell for 2 s. All RNA molecules used in the experiments were renatured by warming to 65°C for 10 min and cooling to room temperature; then, the anti-RNase was added together with carbamoylcholine and MK-801 (Ulrich et al. 1998). The aptamer by itself did not affect the receptor desensitization up to the highest concentration tested (100 μM). Measurements were made within 2 h of preparation of the RNA solution. At a constant concentration of carbamoylcholine (100 μM), the ratio of the maximum current amplitude obtained in the absence (A_O) and presence (A_I) of a constant concentration of MK-801 (500 μM, a concentration at which the receptors are almost completely inhibited) was determined as a function of the concentration of the alleviating aptamer being tested. Similarly, the ratio of the maximum current amplitude in the absence (A_O) and presence (A_I) of various concentrations of aptamers that inhibit the nAChR at constant 100 μM carbamoylcholine was determined.

Data Analysis

Analyses of the data and nonlinear least squares fitting were carried out using Origin 3.5 software according to a method described previously (Udgaonkar and Hess 1987). The curves were fitted using equations **1** and **2** (Hess and Grewer 1998), where A_O and A_I represent the maximum amplitude of the current in cell-flow measurements made in the absence and presence of inhibitor, respectively.

$$A_0/A_I = 1 + ([I]/K_I)\{K_{Apt}/[K_{Apt} + [Apt]]\} \quad (1)$$

$$A_0/A_I = 1 + ([Apt]/K_{Iapt}) \quad (2)$$

The equations assume that the inhibitor and the compound being tested compete for the same binding site. They also assume that the maximum current amplitude obtained in transient kinetic experiments is a measure of the concentration of open receptor channels (Udgaonkar and Hess 1987; Hess and Grewer 1998). [I] is the molar concentration of the inhibitor and K_I its observed dissociation constant. [Apt] is the molar concentration of the aptamer tested, and K_{Iapt} and K_{Apt} are the observed dissociation constants of the inhibitory and alleviating aptamers, respectively.

Secondary Structure Prediction

Zuker's mfold program was used to predict the most stable secondary structures of the RNA aptamers at 25°C and in the presence of 145 mM sodium chloride and 1.2 mM magnesium chloride (Zuker 2003) and to estimate the melting temperature. It can be accessed online from http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi or at http://www.idtna.com/SciTools/SciTools.aspx (Owczarzy et al. 2008). All the sequences are written from 5′ to 3′. The consensus sequences are in uppercase in Fig. 2c.

Results and Discussion

Mechanism of Inhibitor and nAChR Interaction

The proposed mechanism for the action of noncompetitive inhibitors of the nAChR is given in Fig. 1a (Hess and Grewer 1998; Hess et al. 2000). In this cyclic mechanism, the principle of microscopic reversibility (reviewed in Hammes 2000) requires that the ratio of the dissociation constants, $\overline{K_I}/K_I$, of compounds binding to the open- and closed-channel receptor forms, respectively, is equal to the ratio of the channel-opening equilibrium constant in the absence ($\Phi^{-1} = k_{op}/k_{cl}$) and presence ($\Phi_I^{-1} = k^*_{op}/k^*_{cl}$) of the compound that binds to the closed- and open-channel forms of the receptor (Fig. 1a).

Two predictions arise from the cyclic mechanism concerning the effects of noncompetitive molecules on the receptor kinetics (Hess et al. 2000).

1. Ligands that bind with higher affinity to the closed-channel form (RL_2, Fig. 1a) than to the open-channel form ($\overline{RL_2}$, Fig. 1a) shift the channel-opening equilibrium toward the closed-channel form of the receptor and inhibit the receptor.
2. Ligands that bind with equal or higher affinity to the open-channel form than to the closed-channel form can displace the inhibitor but do not affect unfavorably the channel-opening equilibrium. Therefore, they do not inhibit the receptor but can counteract inhibition by displacing the inhibitors that bind with higher affinity to the closed- than to the open-channel form.

RNA aptamers were selected for the cocaine-binding site of the nAChR to test these predictions (Ulrich et al. 1998; Hess et al. 2000). They fell into two classes based on their effects on the activity of the nAChR and on their consensus sequence. Class I RNA aptamers have the consensus sequence UUCACCG (Fig. 2b). They bind with

higher affinity to the closed-channel conformation than to the open-channel conformation and inhibit the nAChR in BC_3H1 cells in the low micromolar concentration range (Ulrich et al. 1998). This class of aptamer supports the first prediction arising from the cyclic mechanism (Fig. 1a) (Hess et al. 2000). Class II RNA aptamers contain the consensus sequence GCUGAA (Fig. 2b) (see supplementary information, Table S2; data obtained by Ulrich et al. 1998). These bind with equal or higher affinity to the open- than the closed-channel form of the nAChR. When tested in the micromolar concentration range, they do not inhibit the nAChR of BC_3H1 cells but can still displace inhibitors that bind with higher affinity to the closed- than the open-channel form of the receptor (Ulrich et al. 1998). They do, therefore, alleviate inhibition of the nAChR by MK-801 or cocaine (Ulrich et al. 1998; Hess et al. 2000). This class of aptamers provides experimental evidence for the second prediction of the cyclic mechanism (Fig. 1a). The DNA template used in the selection of the RNA aptamers is shown in Fig. 2a.

It was of interest to determine the functional sequences within the large RNA aptamers responsible for displacing small organic molecules such as cocaine, MK-801 and PCP (Fig. 1b) from the nAChR. Among the millions of RNA aptamers with different sequences, we obtained 14 class I aptamers and nine class II aptamers, each class having a characteristic consensus sequence of six to seven nucleotides. Does the active part of the RNA molecule exist within the consensus sequence (Fig. 2b)? In assays done with full-length RNA molecules, aptamer I-14 (class I) and aptamer II-3 (class II) had the highest activity in their respective class in inhibiting the receptor or alleviating MK-801 inhibition of the nAChR, respectively (Ulrich et al. 1998; Hess et al. 2000). Accordingly, truncated molecules containing 20 nucleotides (stem loop I-14), 18 nucleotides (stem loop II-3), 21 nucleotides (stem loop II-30) and 19 nucleotides (stem loop II-3′) (Fig. 2c), each containing the consensus sequence of these aptamers, were tested first. In Fig. 2c, the consensus sequences are in uppercase.

All aptamers were tested with BC_3H1 cells expressing the nAChR (Schubert et al. 1974) by using whole-cell current recordings (Hamill et al. 1981) and the cell-flow technique (Udgaonkar and Hess 1987).

Class I Aptamers

Carbamoylcholine (100 μM) was used in the kinetic measurements, a concentration at which the receptors are predominantly in the closed-channel conformation (Niu et al. 1995). Carbamoylcholine is a stable analogue of acetylcholine (Broadley 1996), the natural neurotransmitter for the nAChR. Aptamer I-14, which inhibits the receptor, had a high affinity for the nAChR with a very low apparent dissociation constant, $K_{Iapt} = 0.5$ μM (Ulrich et al. 1998). It was, therefore, chosen for this investigation. We tested first the activity of the part of the aptamer containing the consensus sequence with the chain length reduced to

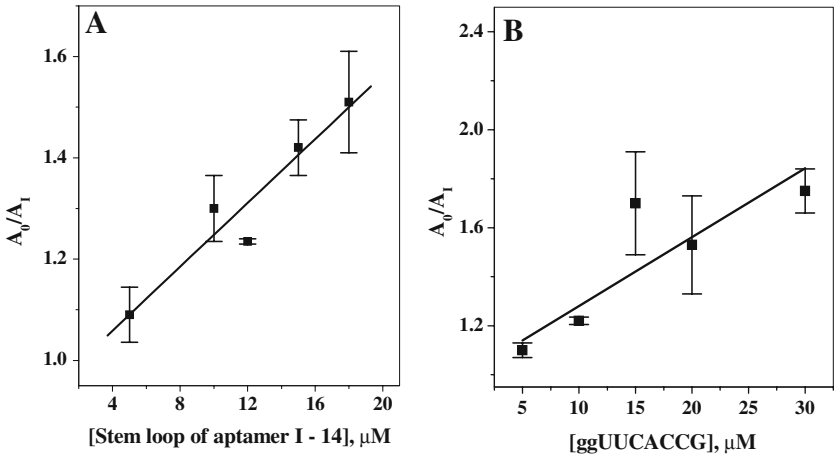

Fig. 3 Cell-flow measurements with the I-14 stem loop and with ggUUCACCG were made at pH 7.4, –60 m*V* and 22°C in bath buffer (composition given in "Materials and Methods"). The concentration (100 μM) of carbamoylcholine was constant in the experiments. Cells were preincubated for 2 s with the truncated class I aptamer being tested. A_O and A_I are the maximum currents obtained in the absence and presence of the truncated aptamer, respectively. Equation 2 (see "Materials and Methods") was used to calculate the apparent dissociation constants from the data shown (Hess and Grewer 1998). **a** Inhibition of the nAChR by the stem loop (Fig. 2c) of class I RNA aptamer I-14. The I-14 stem-loop molecule has an apparent dissociation constant, K_{Iapt}, of 37 ± 3 μM. **b** Inhibition of the nAChR by ggUUCACCG of class I RNA aptamers. This RNA molecule has an apparent dissociation constant, K_I, of 33 ± 4.8 μM. Molecules containing a truncated consensus sequence (gggUUCA and gggACCG) had no apparent activity, up to the highest concentration tested, 40 μM. The guanosine residues at the 5′ end of the transcribed RNA aptamers are added during their biosynthesis by the T7 RNA Polymerase (Jia and Patel 1997). Each data point represents the average of three to six measurements with at least two different cells

only ~ 20% of the full-length aptamer. We refer to this molecule as stem loop I-14 (Fig. 2c). Like its parent molecule, aptamer I-14, it inhibited the nAChR but with a reduction in affinity for the receptor (from 0.5 to 37 ± 3 μM) (Table 1, Fig. 3a).

An inhibitory aptamer containing the consensus sequence plus two guanosine residues (ggUUCACCG) was then tested (Table 1). This molecule inhibited receptor activity (K_{Iapt} = 33 ± 4.8 μM, Table 1), as determined by using the cell-flow technique, even after ~90% of the full-length aptamer sequence was deleted (Fig. 3b). To determine whether the two guanosine residues attached to the consensus sequence during transcription (Jia and Patel 1997) make a difference in the activity of the molecule, the consensus sequence UUCACCG was chemically synthesized (Syngen). The difference in K_{Iapt} values between the consensus sequence with two guanosine residues attached (K_{Iapt} = 33 ± 4.8 μM, Table 1, n = 20 measurements) and the consensus sequence alone (K_{Iapt} = 58 ± 4 μM, Table 1, n = 20 measurements) is marginal ($P < 0.0001$). The affinities of these molecules for the receptor are similar to the affinity of the much larger stem-loop I-14 (Fig. 2c) (K_{Iapt} = 37 ± 3 μM, Table 1, n = 20 measurement, $P <$ 0.0001).

When sequences shorter than the consensus sequence were tested, no activity was detected within the concentration range (up to 100 μM) tested. The RNA ligands gggUUCA and gggACCG were both inactive at 100 μM concentration.

In order to confirm the functional significance of the class I consensus sequence, RNA molecules containing scrambled sequences (CUCAGCG, UCACCG and GCA CCG) were tested. The alteration of the sequence adversely affected the function of the molecule; the scrambled molecules, up to the highest concentration tested (100 μM), did not inhibit receptor function.

Finally, a DNA version (ggTTCACCG) of one of the active truncated inhibitory aptamers (ggUUCACCG) was evaluated. The activity of the DNA analogue was qualitatively similar to the RNA counterpart in inhibiting the nAChR, although the affinity of the DNA analogue for the receptor was less (K_{Iapt} = 74 ± 3 μM, Table 1, n = 16 measurements, $P < 0.0001$). All these results indicate that the consensus sequence is the minimal biologically active sequence motif of the inhibitory class I RNA aptamers.

Class II Aptamers

The alleviatory aptamer II-3 was chosen for this study because among class II RNA aptamers it has the highest affinity for the receptor (Table 1) (Ulrich et al. 1998; Hess et al. 2000). The truncated aptamer, the II-3 stem loop molecule including the consensus nucleotides (Fig. 2c), contains 20% of the parental nucleic acid sequence. Like the full-length class II RNA aptamers, the II-3 stem loop alleviates MK-801 inhibition without itself inhibiting the receptor (Table 1). The activity of the truncated stem loop aptamer was reduced less than 10-fold. The value of the apparent affinity constant K_{Apt} for the full-length aptamer is 0.3 ± 0.15 μM (Hess et al., 2000) and that of the II-3 stem loop is 3 ± 1.4 μM (Table 1, Fig. 4a, n = 16 measurements).

The effect of a molecule containing the consensus sequence of aptamer II-3 and one additional guanosine residue (gGCUGAA) on the reversal of MK-801 inhibition of the nAChR was then determined (Fig. 4b, Table 1). The affinity of the short molecule for the receptor, as measured by its ability to alleviate MK-801 inhibition (K_{Apt} = 4 ± 1.5 μM, Table 1, Fig. 4b, n = 20 measurements), is nearly equal to that of the much larger stem loop of aptamer II-3 (Figs. 2c and 4a, K_{Apt} = 3 ± 1.4 μM, Table 1, $P < 0.05$). An example of the results of a typical cell-flow experiment to determine the activity of the aptamer is shown in Figure 5a. For more quantitative analysis of such results (Figs. 4 and 5b), equation **1** was used (see "Data Analysis" under "Materials and Methods"). As with class I RNA aptamers, the additional guanosine residue(s) does not significantly affect the activity of the truncated class II-3 and II-30 aptamers.

We shortened the sequences still further, to ggUGAA and ggGCUG. In these shortened molecules, 94% of the full-length II-3 (Fig. 2b) aptamer was removed. Of these two RNA molecules only ggGCUG alleviated MK-801-induced inhibition (Fig. 5b). The affinity of ggGCUG is reduced by a factor of ~110 (K_{Apt} = 55 ± 5 μM, Table 1, n = 16 measurements) compared to the full-length aptamer, as determined by its ability to alleviate MK-801 inhibition of the nAChR.

In order to rule out the contribution of the guanosine residues to the activity of the truncated class II aptamers, chemically synthesized RNA sequences (GCUGAA and GCUG) were tested by cell-flow measurements. These aptamers alleviated MK-801 inhibition of the receptor, at 10 μM in the case of GCUGAA and at 100 μM in the case of GCUG (Fig. 5) (Table 1), indicating again that the origin of the biological activity lies in the consensus sequence. Our results are in accordance with previous functional analyses of RNA aptamers for other targets and the important role played by conserved sequences (e.g., Lauhon and Szostak 1995; Kikuchi et al. 2005).

In order to test the class II consensus sequence–activity relationship, the shortest active sequence, GCUG, was scrambled, giving UCGG. The scrambled sequence neither affected the nAChR function nor alleviated MK-801 inhibition of the receptor, up to the concentration tested, 100 μM. This finding indicates again that consensus sequences

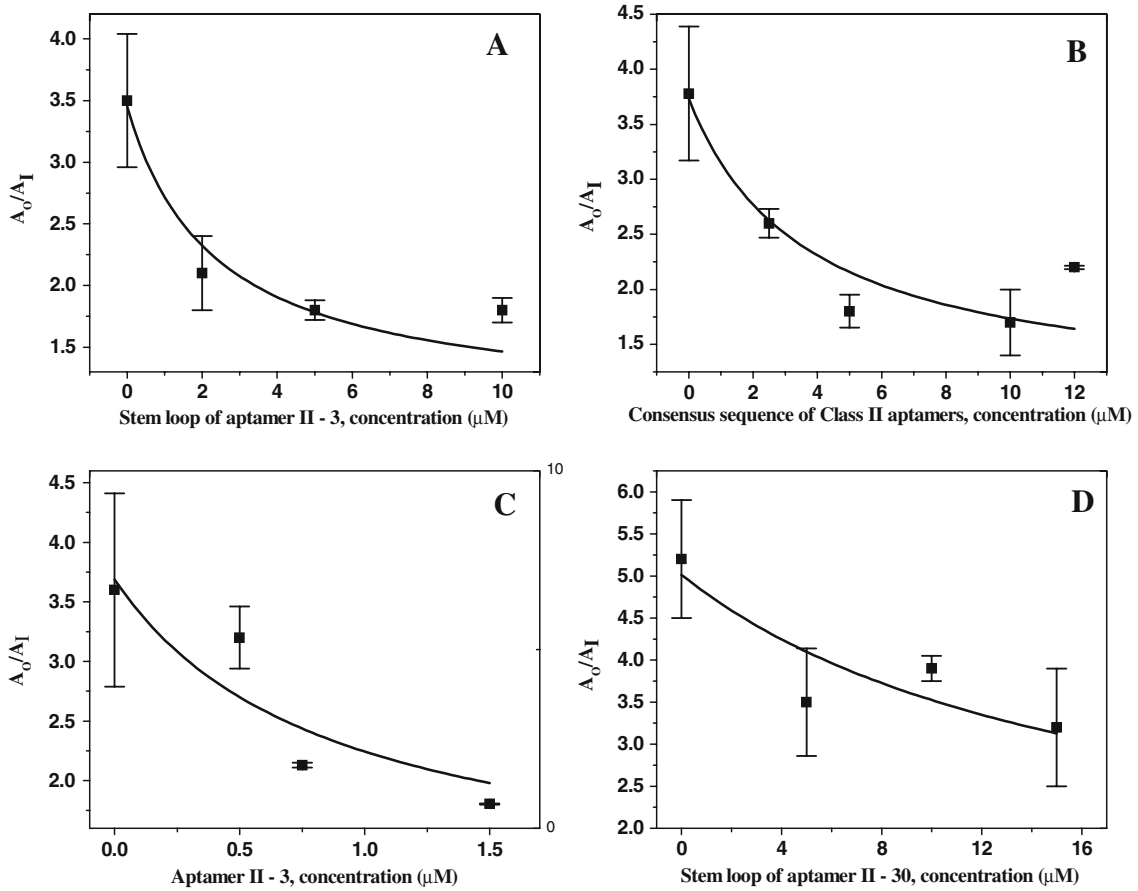

Fig. 4 Cell-flow measurements with truncated class II aptamers were made at pH 7.4, –60 m*V* and 22°C in bath buffer (composition given in "Materials and Methods"). The concentration of carbamoylcholine (100 μM) was constant in the experiments. Cells were preincubated for 2 s with the various concentrations of the aptamers indicated. A_O and A_I are the maximum currents obtained in the absence and presence of 500 μM MK-801, respectively. Equation 1 (see "Materials and Methods") was used to determine the apparent dissociation constants of the aptamers tested (Hess et al. 2000). **a** Alleviation of MK-801 inhibition of the nAChR by the II-3 stem loop (Fig. 2c). The stem-loop molecule has an apparent dissociation constant, K_{Apt}, of 3 ± 1.4 μM. Each data point represents the average of three to six measurements with at least two different cells. **b** Alleviation of MK-801 inhibition of the nAChR by the gGCUGAA aptamer (Fig. 2b). The aptamer has an apparent dissociation constant, K_{Apt}, of 4 ± 1.5 μM. **c** Alleviation of MK-801 inhibition of the nAChR by the II-30 aptamer. The aptamer has an apparent dissociation constant, K_{Apt}, of 0.8 ± 0.45 μM. **d** Alleviation of MK-801 inhibition of the nAChR by the II-30 stem loop (Fig. 2c). The aptamer has an apparent dissociation constant, K_{Apt}, of 16 ± 8 μM

play a critical role in the biological function of the aptamers and that altering the sequence perturbs their function. Since the shortest functional aptamer tested (GCUG) shows activity at 100 μM, this concentration was chosen as the empirical criterion for classifying RNA molecules into active or inactive in this work.

Does the DNA version (gggGCTGAA) of one of the short, active class II RNA aptamers have biological activity? We determined that it alleviated MK-801 inhibition of nAChR with a dissociation constant, K_{Apt}, of 15 ± 5 μM ($n = 20$ measurements).

Having established that the activity of the aptamers results from their consensus sequence, we investigated another alleviating aptamer (II-30) with the same consensus sequence as aptamer II-3 (Fig. 2b) (supplementary information Table S2, from Ulrich et al. 1998). It also alleviated MK-801 inhibition of the nAChR. The value of its apparent dissociation constant, K_{Apt} (0.8 ± 0.45 μM, Table 1, Fig. 4c, $n = 12$ measurements), is similar to that of aptamer II-3 (0.3 ± 0.15 μM) (Hess et al. 2000).

As with aptamer II-3, the short stem loop of aptamer II-30 (Fig. 2c) containing the consensus sequence was tested. This stem loop was also active (Fig. 4d, Table 1). The K_{Apt} value for the II-30 stem loop (16 ± 8 μM, $n = 12$ measurements) is higher than that for the II-3 stem loop ($K_{Apt} = 3 \pm 1.4$ μM, $P < 0.0001$). Since both aptamer II-3 and aptamer II-30 have the same consensus sequence, the difference in activity indicates a contributory effect from the nucleotides surrounding the consensus sequence.

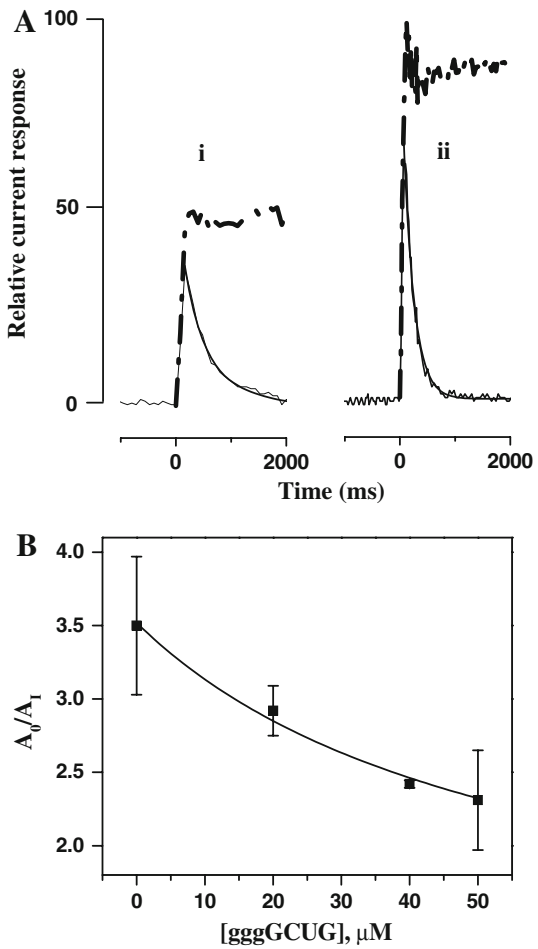

Fig. 5 Cell-flow measurements with ggGCUG were made at pH 7.4, –60 m*V* and 22°C in bath buffer (composition given in "Materials and Methods"). The concentration of carbamoylcholine (100 μM) was constant in the experiments. Cells were preincubated with the aptamers for 2 s. A_O and A_I are the maximum currents obtained in the absence and presence of 500 μM MK-801, respectively. Equation 1 (see "Materials and Methods") was used to determine the apparent dissociation constants of the aptamers (Hess et al., 2000). **a** Typical traces from cell-flow experiments with BC_3H1 cells containing the muscle-type nAChR, in the presence of 500 μM MK-801 in the absence (i) and presence (ii) of 40 μM class II aptamer ggGCUG (Table 1). The relative current response obtained with 100 μM carbamoylcholine, which is typically 1–5 nAmps, is plotted against the time during which ligands flowed over the cell. The trace parallel to the abscissa represents the current amplitude corrected for receptor desensitization (Udgaonkar & Hess, 1987) that occurs during the rising phase of the current. **b** Alleviation of MK-801 inhibition of the nAChR by the minimal sequence of a class II RNA aptamer, ggGCUG. The apparent dissociation constant of the aptamer, K_{Apt}, is 55 ± 5 μM. Two guanosine residues were added at the 5′ end of the transcribed RNA aptamer by T7 RNA polymerase (Jia & Patel, 1997), which is used in the synthesis of the aptamer (see "Materials and Methods"). Each data point represents the average of three to six measurements with at least two different cells. Equation 1 was used to analyze the data

As the assays carried out with both classes of RNA aptamers indicated that the consensus sequences of the aptamers are responsible for the activities, we also prepared a truncated RNA molecule (II-3′ stem loop) with the class II consensus sequence (GCUGAA) flanked by Watson-Crick complementary bases (Fig. 2c). The motive behind this design was to develop a truncated RNA aptamer with a predicted (Zuker 2003) conformation containing the active sequence. This was done to investigate the effect of the neighboring nucleotides on the aptamer activity due to the consensus sequence. Interestingly, although the II-3′ stem loop (Fig. 2c) contains the consensus sequence, the molecule does not alleviate MK-801 inhibition up to the highest concentration tested, 100 μM. This demonstrates that while the consensus sequence is necessary for biological activity, the neighboring nucleotides can have a significant modulating effect. The result also indicates that in the full-length aptamer the conformation of an RNA sequence is different from that indicated in the truncated stem-loop II-2 aptamers in Fig. 2c.

Conclusions

A 90% reduction in the sequence lengths of inhibitory and alleviatory aptamers did not abolish the effect of the aptamers on the receptor, although their activity was reduced, as measured by their ability to inhibit (class I) or alleviate (class II) inhibition (Table 1). All seven nucleotides of the class I consensus sequence are required for biological activity, but only four of the six nucleotides in the class II consensus sequence are required. The nucleotides of the full-length aptamers have a considerable influence on the biological activity. As indicated by the dissociation constants measured electrophysiologically, the biological activity of the shortest fragment of class I aptamers is reduced by a factor of ~50 and that of the shortest fragment of class II aptamers by a factor of ~150 compared to the full-length class I ($K_{Iapt} = 0.5$ μM) and class II ($K_{Apt} = 0.3$ μM) aptamers. Interestingly, a short 19-nucleotide fragment of a class II aptamer containing the consensus sequence (II-3′ stem loop) was biologically inactive in our test, indicating the importance of the three-dimensional structure of the RNA polymer. The techniques for producing large nucleotide polymers that bind with high affinity to specific sites on biomolecules (Tuerk and Gold 1990; Ellington and Szostak 1990) together with the possibility that only short sequences are required for their biological activity may eventually become useful in developing compounds that counteract dysfunctional biological molecules. Whether the results apply only to the muscle nAChR and its interaction with the specific ligands used in the investigations described here is an interesting question to be addressed in future studies.

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