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Selection of 2′-Fluoro-modified RNA Aptamers for Alleviation of Cocaine and MK-801 Inhibition of the Nicotinic Acetylcholine Receptor

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Abstract. The nicotinic acetylcholine receptor (nAChR) belongs to a group of five stracturally related proteins that regulate signal transmission between approximately 10^{12} cells of the mammalian nervous system. Many therapeutic agents and abused drugs inhibit the nAChR, including the anti-convulsant MK-801 and the abused drug cocaine. Many attempts have been made to find compounds that prevent inhibition by cocaine. Use of transient kinetic techniques to investigate the inhibition of the receptor by MK-801 and cocaine led to an inhibition mechanism not previously proposed. The mechanism led to the development of combinatorially synthesized RNA ligands that alleviate inhibition of the receptor. However, these ligands are relatively unstable. Here we determined whether much more stable 2′-fluoro-modified RNA ligands can be prepared and used to study the alleviation of receptor inhibition. Two classes of 2′-fluoro-modified RNA ligands were obtained: One class binds with higher affinity to the cocaine-binding site on the closed-channel form and, as predicted by the mechanism, inhibits the receptor. The second class binds with equal or higher affinity to the cocaine-binding site on the open-channel form and, as predicted by the mechanism, does not inhibit the receptor, and does alleviate cocaine and MK-801 inhibition of the nAChR. The stability of these 2′-fluoro-RNAs expands the utility of these ligands.

Key words: 2′Fluoro-modified RNA aptamers — Nicotinic acetylcholine receptor — MK-801 — Cocaine–SELEX

Introduction

The nicotinic acetylcholine receptor (nAChR) is a member of the superfamily of membrane proteins that regulate intercellular signal transmission between approximately 10^{12} cells of the mammalian nervous system (Katz & Thesleff, 1957; Kandel et al., 1995). Many therapeutic agents and abused drugs inhibit the nAChR, including the local anesthetic procaine, the anticonvulsant MK-801 [(+)-dizocilipine], toxins such as philanthotoxin (Jayaraman, Thiran & Hess, 1999), α-bungarotoxin and cobratoxin, as well as the abused drugs cocaine and phencyclidine (PCP) (Niu & Hess, 1993; Niu, Abood & Hess, 1995; Miwa et al., 1999).

Cocaine (*see* Fig. 2A) is abused widely in the world. There are 14 million users of abused drugs in the United States (NIH, 1997). Cocaine abuse can result in cardiac and respiratory disease, stroke, seizure, sudden death and neurological disorders (Karch, 1999). Cocaine inhibits the nAChR (Heidman & Changeux, 1978; Niu et al., 1995), which plays a major role in controlling communication between cells of the nervous system, and it also inhibits the dopamine transporter and possibly other transporter proteins (Kuhar, Ritz & Boja, 1991; Giros et al., 1996). No effective treatment for a cocaine overdose exists (Hahn & Hoffman, 2001). Significant advances have been made in the use of enzymes and catalytic antibodies to decrease the concentration of cocaine in the blood stream (Lerner & Janda, 1995; Xie et al., 1999). Few advances have been made regarding the direct protection of transporters and neurotransmitter receptors such as the nAChR.

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; nAChR, nicotinic acetylcholine receptor; SELEX, systematic evolution of ligands by exponential enrichment; nt, nucleotide; PCP, 1-phenyl-cyclohexylpiperidine; TBE, Tris-borate-EDTA

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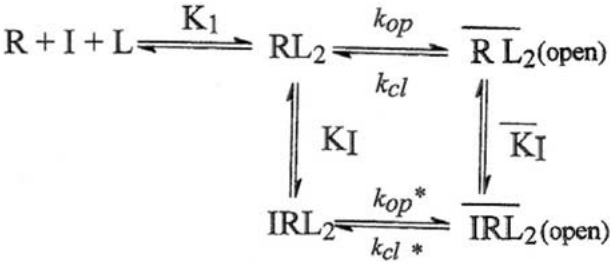


Fig. 1. Proposed mechanisms for the inhibition of the nAChR by cocaine. The upper line represents the minimum mechanism for the opening of the receptor channel (Katz & Thesleff, 1957). Receptor R binds the neurotransmitter L. RL_2 represent the closed-channel conformations. RL_2 represents the open-channel conformation of the receptor that allows inorganic cations to cross the cell membrane. K_I 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 (Hess & Grewer, 1998); in presence of inhibitor; Φ_I^{-1} is k_{op*}/k_{cl*}. The reactions shown occur in the microsecond to millisecond time region (Hess & Grewer, 1998). For clarity, the desensitization reaction, which in the case of the nAChR occurs in the 100–500-ms time region (Hess, Cash & Aoshima, 1979; Hess & Grewer, 1998) and the binding of the inhibitor I to the unliganded or monoliganded receptor forms are not shown. The relatively slow transitions of receptor/inhibitor complexes to non-conductive forms (Grewer & Hess, 1999) are also not shown. The proposed cyclic inhibition mechanism involves complexes of the inhibitor with the open-channel conformations in which the open channel is not blocked by the inhibitor (i.e., it conducts ions). This minimum mechanism is based on chemical kinetic measurements (Grewer & Hess, 1999; Jayaraman, Usherwood & Hess, 1999). The principle of microscopic reversibility requires that the ratio $K_I/\overline{K_I} = \Phi^{-1}/\Phi_I^{-1}$ where K_I is the dissociation constant of the inhibitor from the closed-channel form RL_2 and K_I the dissociation constant of the inhibitor from the open-channel form RL_2. Therefore, compounds that bind to a regulatory site with higher affinity for the closed-channel conformation than 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 not expected to change the channel-opening equilibrium constant unfavorably. These compounds are, therefore, expected to displace inhibitors from the regulatory sites without inhibiting receptor activity (Hess et al., 2000).

The mechanism of inhibition of the nAChR by cocaine and the anticonvulsant MK-801 has been investigated extensively during the last two decades by measuring the binding of radio-labeled inhibitors (Eldefrawi et al., 1980), by single-channel current recordings (Neher & Sakmann, 1976; Neher, Sakmann & Steinbach, 1978), and by rapid kinetic techniques (Milburn et al., 1988). The adoption of transient kinetic techniques (Udgaonkar & Hess, 1987; Milburn et al., 1988) led to a mechanism for receptor inhibition not previously proposed (Hess et al., 2000) (Fig. 1). The measurements thus made indicated that the mouse embryonic muscle nAChR in BC_3H_1 cells has two binding sites for MK-801 and cocaine that equilibrate with inhibitors in two different time regions, 50 ms and 1 s (Hess et al., 2000). Only the mechanism involving the rapidly equilibrating inhibitory site (Fig. 1) is considered here. Two types of ligands can bind to the rapidly equilibrating allosteric site of the nAChR (Hess et al., 2000). Class I ligands, including cocaine and MK-801, bind to a regulatory site on the closed-channel conformation of the nAChR with higher affinity than to the site on the open-channel form and shift the equilibrium toward the closed-channel form, thereby inhibiting the receptor. (ii) Class II ligands, including cocaine analogs (Hess et al., 2000; 2003; Chen, Banerjee & Hess, 2004), bind to a regulatory site with an affinity for the open-channel conformation equal to or higher than their affinity for the closed-channel conformations and, therefore, do not shift the equilibrium towards the closed-channel form. These ligands, therefore, do not inhibit the receptor but are able to displace inhibitors (Hess et al., 2000) (Fig. 1).

Unmodified RNA ligands for the nAChR that were prepared previously (Ulrich et al., 1998) are extremely sensitive to nucleases commonly found in cells, tissues and body fluids (Pieken, 1991). This limits their use in investigations of the mechanism of alleviation of receptor inhibition with cells or nervous system tissue or as diagnostic and therapeutic agents. The modification of natural RNAs with fluoro or amino groups at the 2′-hydroxy position in the pyrimidine nucleotides can generate nuclease-resistant random oligonucleotide libraries (Pieken, 1991; Lin et al., 1994). The 2′-fluoro or amino-2′-deoxypyrimidine triphosphates can be incorporated into RNA by T7 RNA polymerase in the SELEX steps (Aurup, Williams & Eckstein, 1992). With such modifications, the half-life of RNA aptamers in 95% of mammalian fluids increases from approximately 8 seconds for unmodified RNA to 86 hours for fluoro-modified RNA and 174 hours for amino-modified RNA (Pagratis et al., 1997). The fluoro-modified RNA libraries have more rigid structures and, in general, higher affinities for their target than do amino-modified RNA ligands (Pagratis et al., 1997).

Here we determine whether 2′-fluoro-modified RNA ligands can be prepared that bind with different affinities to the cocaine-binding site on the closed- and open-channel forms of the nAChR. The results show that most of the stable 2′-fluoropyrimidine RNA aptamers, with considerably more stability in biological fluids than unmodified RNA aptamers, exhibit a similar high affinity for the cocaine-binding site than do the unmodified RNA aptamers (Ulrich et al., 1998; Hess et al. 2000). Two classes of fluorinated RNA aptamers were identified; Class I 2′-fluoro-RNA aptamers bind with higher affinity to an allosteric site on the closed-channel conformation than the open-channel conformation. As expected from the mechanism (Fig. 1) of receptor inhibition (Hess et al., 2000), in submicromolar concentrations

they inhibit the receptor. Class II 2′-fluoro-RNA aptamers bind with higher affinity to an allosteric site on the open-channel conformation and as expected from the mechanism (Fig. 1) of receptor inhibition (Hess et al., 2000), they alleviate inhibition of the nAChR induced by 200 μM cocaine or 500 μM MK-801. Alleviation requires only a low micromolar concentration of the Class II aptamer. Because these aptamers were selected by displacing them from the membrane-bound nAChR by 1-phenyl-cyclohexylpiperidine (PCP), which binds to the same site on the nAChR as does cocaine (Karpen & Hess, 1986), we assume that the selected aptamers and cocaine bind to the same site on the receptor. This type of aptamer is, therefore, expected to be also useful not only in mapping the regulatory sites of the nAChR with different subunit compositions (Hess et al., 2003) but also to identify the cocaine-binding sites on the transporter proteins and other neurotransmitter receptors in cells and nervous system tissues.

Materials and Methods

Materials

A nAChR-containing membrane fraction prepared (Szczawinska et al., 1992) from frozen *Torpedo californica* electric organs (Pacific Bio-Marine, Venice, CA), which are a very rich source of the muscle-type nAChR (Szczawinska et al., 1992), was used. BC_3H_1 cells expressing the muscle fetal type nAChR (Schubert et al., 1974) were purchased from the American Type Tissue Culture Collection.

Carbamoylcholine, MK-801 [(+)-dizocilpine] and other chemicals were purchased from Sigma. [^{125}I] α-bungarotoxin (104-128 Ci/mmol) and [^{3}H] cocaine (50 Ci/mmol) were obtained from New England Nuclear (Beverly, MA), and [α^{32}P]-ATP (3,000 Ci/mmol) from Amersham Pharmacia Biotech (Piscataway, NY). 2′-Fluoro-2′ deoxyuridine-5′-triphosphate (2′F-dUTP) and 2′-fluoro-2′-deoxycytidine-5′-triphosphate (2′F-dCTP) were purchased from TriLink BioTechnologies (San Diego, CA). The sources of other materials are noted in the relevant section.

Preparation of nAChR Membrane Fragments and Determination of Receptor Concentration

The membrane preparations were a gift from Professor Richard Hann, Professor Vesna Eterovic, and Oné Pagan (Universidad del Central Caribe). The procedure for the preparation of the nAChR-rich membrane fragments has been described (Szczawinska et al., 1992; Ulrich et al., 1998). The concentration of the receptor in the membrane was determined by using [^{125}I] α-bungarotoxin binding as described by Schmidt and Raftery (1973) and ranged between 0.5 and 1.2 nmol α-bungarotoxin sites per mg of membrane protein.

DNA Template and PCR Primer Synthesis

An 87-nt oligonucleotide and two primers were synthesized by Keystone Labs, Biosource International (Camarillo, CA). The sequence of the oligonucleotide is the same as that described by Fitzwater and Polisky (1996). This single-stranded oligonucleotide contains three different regions, a 40-nt central randomized region and two constant regions, 5′-GCC-TGT-TGT-GAG-CCT-CCT-GTC-GAA-, that precede and follow the 40 random nucleotides, TT-GAG-CGT-TTA-TTC-TTG-TCT-CCC-3′. The sequences of the two primers for extension and amplification of this template were as follows:
Primer-24: 5′-GCC-TGT-TGT-GAG-CCT-CCT-GTC-GAA-3′,
Primer-40: 5′-TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-AA G-AAT-AAA-CGC-TCA-A-3′. Primer-40 contained a T7 RNA polymerase promoter for transcription of dsDNA in the SELEX cycles (Fitzwater & Polisky, 1996).

Single-Stranded DNA Template Pool Extension and Amplification

To generate dsDNA with various sequences from the synthetic ssDNA pool, a large-scale Klenow fragment fill-in reaction was carried out (Marshall & Ellington, 2000). A solution containing 9 μl of annealing buffer (consisting of 100 mM Tris-HCl, pH 8.0 and 100 mM $MgCl_2$), 9.5 μl 2 nmol gel-purified Primer-40, 1 nmol 2 μl template DNA, and 69.5 μl double-distilled H_2O (dH_2O) were vortexed for 20 s, heated to 95°C for 5 min, and placed on ice for 5 min. Sixty μl of a 10-fold stock solution of the Klenow buffer (100 mM Tris-HCl, pH 7.5, 75 mM dithiothreitol (DTT), 30 μl 10 mM dNTPs (2′-deoxynucleoside 5′-triphosphate), 6 μl Exo-Klenow (New England Biolab), and dH_2O were added to give a total volume of 600 μl. The reaction solution was incubated at 37°C for 45 min. The reaction was stopped by adding 12 μl 100 mM EDTA, pH 8.3, and the solution was concentrated in a Nanosep 30 k microconcentrator (PALL German Laboratory, Ann Arbor, MI) according to the manufacturer's instructions.

The ssDNA pool was amplified from the original pool by PCR for 5 cycles in the following way. The total volume in the PCR reaction was 28.8 ml (a total of 288 vials, 100 μl per vial). Each PCR reaction (100 μl) contained 10 μl PCR buffer (Gibco), 1.5 mM $MgCl_2$, 200 μM dNTP, 100 pmol Primer-40 and Primer-24, 2 μl DNA template (0.2-0.5 μg), 2.5 U Taq DNA polymerase and was brought to a final volume of 100 μl with dH_2O. The RNA in the reaction mixture was denatured first at 95°C for 3 min, and then cycled five times as follows: 95°C for 3 min, annealed at 55°C for 50 s, and elongated at 72°C for 2 min. A final extension cycle was run at 72°C for 10 min.

In general, an initial ssDNA pool prepared by chemical synthesis will produce only 10–30% full-length dsDNA (Marshall & Ellington, 2000). It is, therefore, necessary to purify and amplify the extended product to get the correct size pool. (Marshall & Ellington, 2000). The purified, randomized dsDNA was used as the final template for the transcription of the modified RNA pool.

Transcription of Stable RNA Using 2′-Fluoropyrimidines

Transcription of the PCR product was performed using a T7-MEGAshortscript in vitro Transcription Kit for large-scale synthesis of RNA (Ambion, Austin, TX). For in vitro transcription, unmodified CTP and UTP were replaced with 2′-fluoro-2′-deoxycytidine (2′-F-dCTP) and 2′-fluoro-2′-deoxyuridine (2′F-dUTP) to generate a 2′-F-pyrimidine RNA pool (Marshall & Ellington, 2000). The 200 μl transcription reaction mixture contained 20 μl 10× Transcription Buffer (Ambion), 6.8 μl ATP (75 mM), 6.8 μl GTP (75 mM), 25 μl F-dCTP (20 mM), 25 μl F-dUTP (20 mM), 1–4 μg template DNA, and 20 mM T7-MEGAshortscript Enzyme Mix

(Ambion); the final volume was brought to 200 μl with RNAse-free dH_2O. After all the components were added, the reaction mixture was incubated at 37°C for 3 h. To remove the template DNA, 10 μl RNase-free DNase I (2 U/μl) (Ambion, Austin, TX) were added to the reaction mixture, mixed thoroughly by flicking the reaction tube, and the reaction mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 150 μl RNase-free dH_2O and 150 μl 5 M ammonium acetate precipitation solution (pH 5.2) (Ambion). The solution was then extracted using first a 1:1 volume ratio of phenol-chloroform equilibrated with 10% 3 M sodium acetate, pH 5.2. The supernatant was collected and then extracted with 1:1 volume ratio of chloroform. The product was precipitated with 3 volumes of ethanol in the presence of 100 mM ammonium acetate, pH 5.2 and resuspended in 200 μl RNAse-free dH_2O. Ten percent of the reaction product was run on an 8% denaturing polyacrylamide gel for analysis. The gel was run at 250 V for 40 min and checked by UV-shadowing for the integrity of the RNA. The concentration of the product was determined by spectrophotometric absorbance at 260 nm using the approximation that an OD reading of 1 is equal to 40 μg/ml RNA (Sambrook, 1989).

Nitrocellulose Filter-Binding Selection

A nitrocellulose filter-binding technique was used to select RNA ligands that specifically bound to the cocaine-binding site on the nAChR (Ulrich et al., 1998). Briefly, after incubation of the 2′-F-RNA pool (50 μM) with membrane-bound receptor (500 nM) and 2.5 μg/μl tRNA (to reduce unspecific binding), 1 ml of the binding reaction mixture was passed through a filter holder with a 25-mm diameter nitrocellulose membrane disc (Schleicher & Schuell, Keene, NH). The membrane-bound nAChR:2′-F-RNA complex bound strongly to the filter. After washing the complex bound to the filter three times, each with 10 ml external BC_3H_1 buffer (in mM: 145 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$ and 25 HEPES, pH 7.4.), the nAChR:2′-F-RNA complex retained on the filter was incubated in 100 μl of a 1 mM 1-phenyl-cyclohexylpiperidine (PCP) solution (Ulrich et al., 1998). PCP binds to the same site on the nAChR as cocaine does (Karpen & Hess, 1986). One hundred μl of the solution containing RNA ligands displaced by PCP were collected and extracted with 100 μl phenol-chloroform-isoamylalcohol (25:24:1). The supernatant of the solution was extracted with 100 μl chloroform. Ethanol was added to obtain a final concentration of 80% to precipitate the RNA. The RNA was then used as the template for the next round of reverse transcription and PCR.

Gel-Shift Selection

Only nitrocellulose filter-binding selection was used during SELEX rounds 1–3. In the subsequent rounds, both gel-shift selection and filter-binding techniques were performed in turn (Ulrich et al., 1998). For the gel-shift procedure, the [^{32}P]-ATP labeled 2′-F-RNA pool was generated by an in vitro transcription reaction, as described above, in the presence of 50 μCi [α^{32}P]-ATP. The purified RNA pool was diluted to a final concentration of 30 μM in the assay buffer, the external BC_3H_1 buffer containing (in mM) 145 NaCl, 5.3 KC1, 1.8 $CaCl_2$, 1.2 $MgCl_2$ and 25 HEPES at pH 7.4, heated to 70°C for 10 min, and then left at room temperature for 30 min. The 2′-F-RNA was then incubated with *T. californica* membranes that contained 100 nM nAChR as determined by ^{125}I-α-bungarotoxin binding. The total volume of the binding reaction was 80 μl. After incubation, 5 μl gel-loading buffer containing 5% glycerol and 0.04% bromophenol blue, in Tris-Borate-EDTA buffer (100 mM Tris-base, 90 mM boric acid, 1 mM EDTA; TBE), pH 7.2, were added to 10 μl of the binding reaction, and the mixture was loaded onto a 3% native polyacrylamide gel in TBE equilibrated to pH 7.4. The gel was run at 100 V for approximately one hour, and was imaged using a Fuji phosphoscreen and a Molecular Dynamics STORM phosphorimager. The band containing the nAChR:2′-F-RNA complex stained by ethidium bromide (Ulrich et al., 1998) was excised and placed in a tube containing 300 μl elution buffer (0.5 mM sodium acetate, pH 5.2, 2 mM EDTA) and incubated on a horizontal rotating mixer for 12 h at 37°C. The polyacrylamide was pelleted by centrifugation at 1000 × *g* for 10 min, the supernatant containing the nAChR:2′-F-RNA was collected, and the filtrate was extracted with an equal volume of a phenol-chloroform-isoamylalcohol solution at pH 5.2. The supernatant was extracted with an equal volume of chloroform, and precipitated with 80% ethanol as described above. This 2′-F-RNA isolated from the gel was used in the filter-binding selection that followed.

RT-PCR of Selected RNA Pools

One-step 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 containing 0.4 mM dNTP, 2.4 mM $MgSO_4$ and 100 pmol Primer-40, 100 pmol Primer-24, 1 μl RT/Platinum Taq Mix, and 0.1–1μg 2′-F-RNA; the volume was brought to 50 μl with nuclease-free dH_2O. The cDNA synthesis was achieved in a 30 min incubation at 55 °C, and predenaturation of PCR at 94 °C for 2 min was then performed. Then the PCR reaction was run 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 carried out at 72 °C for 10 min. The RT-PCR products were analyzed on an 8 % non-denaturing polyacrylamide gel. If multiple or smeared bands appeared in the gel, the product was purified by cutting out the band and extracting it with phenol-chloroform-isoamylalcohol solution at pH 5.2.

Cloning and Sequencing of Selected RNA Pool

Freshly purified PCR product was cloned using the Invitrogen pCR-4 TOPO TA Cloning Kit. This kit allows one to insert directly 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 TOP 10 competent *E. coli* cells (Invitrogen protocol), and the transformed cells were plated. After incubation at 37 °C for 24 h, 80 colonies were isolated and cultured in Luria-Bertani medium (Sambrook, 1989), and the plasmids were extracted and purified, and then sequenced by the Cornell University Biotechnology Resource Center's sequencing facility.

Determination of Aptamer Consensus Sequences and Prediction of Aptamer Secondary Structures

Seventy individual aptamers were obtained by cloning and sequencing (*see* above). The previously random sequences were aligned and searched for consensus motifs by using the "FIND" function of a word processing program (Microsoft Word 2000) and by using a DNA alignment program (Pearson & Lipman, 1988). RNA secondary structures were predicted by using the MFOLD software (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) (Zuker, 1999). Stem-loop regions, as shown in Fig. 4, were identified and then searched for consensus motifs.

Affinity of Selected RNA for the Cocaine-Binding Site on nAChR

To determine the affinity of the selected pool and of individual aptamers for the nAChR, displacement of [^{3}H] cocaine by 2′-F-RNA was measured. Membrane fragments containing 100 nM nAChR were resuspended in BC_3H_1 external buffer. 100 nM [^{3}H] cocaine was dissolved in 500 μl BC_3H_1 external buffer (Schubert et al., 1974). The reaction mixture containing nAChR, [^{3}H] cocaine, and different concentrations of 2′-F-RNA was incubated at 37 °C for 2 h. The reaction was terminated by filtration of 0.5 ml of the mixture through Whatman GF/F filters on a 10-sample filtration manifold (Hoefer FH225). The filters were washed twice with 2 ml ice-cold BC_3H_1 external buffer. The radioactivity retained on the filters was counted by liquid scintillation counting.

Characterization of Individual RNA Aptamers by Cell-Flow Whole-Cell Current-Recording Measurement

In order to evaluate the effect of individual aptamers on the nAChR, the whole-cell current-recording technique (Hamill et al., 1981) was used in combination with the cell-flow technique (Udgaonkar & Hess, 1987; Hess, 1996), which has a 10 ms time resolution. The BC_3H_1 cell line expressing the fetal muscle-type nAChR used for all the cell-flow experiments was cultured as described by Schubert and colleagues (Schubert et al., 1974). Currents were recorded by using the whole-cell configuration (Hamill et al., 1981) at room temperature, – 60 mV, and pH 7.4. The solution in the recording pipette (intracellular buffer) contained (in mM) 140 KCl, 10 NaCl, 2 $MgCl_2$, 1 EGTA, and 25 HEPES, adjusted to pH 7.4. The BC_3H_1 external buffer solution contained (in mM) 145 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$ and 25 HEPES at pH 7.4. BC_3H_1 cells expressing the nAChR were equilibrated with 100 μM carbamoylcholine in the absence and presence of cocaine and a varying concentration of aptamer or a mixture of aptamers. The cells were preincubated for 2 s with the aptamer solution before the addition of carbamoylcholine alone or carbamoylcholine in the presence of cocaine. The recording electrode was filled with intracellular buffer solution (*see above* for details), and cells were held at a constant transmembrane voltage of – 60 mV. Whole-cell currents were amplified by using an Axopatch 200B (Axon Instruments) amplifier and filtered at 0.5–2 kHz by using a low-pass Bessel filter incorporated in the amplifier. All aptamers were first tested, by recording whole-cell currents, for inhibition of the nAChR after pre-incubation with 0.5 μM cloned aptamer for 2 s. The aptamers that did not inhibit the receptor were further tested for alleviation of cocaine inhibition. For this, a cell was pre-incubated with increasing concentrations of aptamers for 4 s prior to the measurements in the presence of 200 μM cocaine or 500 μM MK-801 as an inhibitor (Hess et al., 2000). MK-801 binds to the same site as cocaine does (Amador & Dani, 1991). The aptamer inhibition curve was fitted by the equation $A/A_{apt} = 1 + [\text{aptamer}]/K_{apt}$, (Hess et al., 2000), where A is the observed current amplitude in the absence of aptamer, and A_{apt} is the observed current amplitude in the presence of aptamer. K_{apt} is the observed dissociation constant of the inhibitor.

The data for the aptamers that did not inhibit the nAChR and that were tested for their alleviation of cocaine or MK-801 inhibition of the nAChR was fitted to the equation (Hess et al., 2000):

$$A/A_I = 1 + ([I]/K_I)(K_{apt}/(K_{apt}+[\text{aptamer}]))$$

A_I is the observed current amplitude in the presence of cocaine or MK-801, $[I]$ is the concentration of the inhibitor cocaine or MK-801, K_I is the apparent dissociation constant of the inhibitor (cocaine or MK-801), and K_{apt} is the observed dissociation constant of an aptamer that displaces cocaine or MK-801.

Stability of 2′-F-Modified Aptamers in Human Serum and Urine

To compare the stability of 2′-F-modified RNA with unmodified RNA, both types of RNAs were synthesized with [α-^{32}P]-ATP in place of non-radioactive ATP during transcription. The unmodified RNA aptamer 51 was synthesized by using unmodified nucleotides. One μl radiolabeled RNA was incubated with 9 μl of either human serum or human urine at 37 °C for 4 h or more. Samples were extracted with phenol and chloroform and the RNA was precipitated with 3 equal volumes of dehydrated ethanol. The precipitated RNA aptamers were run on a denaturing 8 % polyacrylamide gel at 250 V for 30 min and the radioactive bands of the full-length RNA were quantified using a Fuji phosphorimager.

Results and Discussion

Selection of 2′-F-RNA aptamers that bind with high affinity to the cocaine-binding site on the nAChR was initiated with a random RNA pool containing 2′-fluoro-modified pyrimidines (Fig. 2*D*). The 2′-F modified RNA was in vitro transcribed from the dsDNA random pool containing approximately 10^{13} different sequences. These modifications were introduced in order to select for nuclease-resistant aptamers that have potential use in experiments with cells or tissue, for in vivo applications and possible eventual therapeutic use. A variety of possible strategies for creating stable RNA molecules exist. We chose the 2′-F-modification for RNA stabilization as both 2′F-dCTP and 2′F-dUTP can be incorporated enzymatically by T7-RNA polymerase into the transcripts and the resulting 2′F-RNAs used as templates by AMV-reverse transcriptase for cDNA synthesis (Pieken, 1991). Although the yield of the 2′-F-RNA is lower than that obtained by an in vitro transcription reaction in the presence of natural nucleotides, the diversity of the pool is enough for its subsequent selection.

The nAChR used in the selection was obtained from nAChR-rich *T. californica* electric organ membrane (Szczawinska et al., 1992; Ulrich et al., 1998). In a displacement step, a receptor inhibitor was used to obtain the desired RNA molecules that bind to their target site on the nAChR. PCP (1-phenyl-cyclohexylpiperidine), an abused drug (Fig. 2*B*), inhibits nAChR function and competes with cocaine (Fig. 2*A*) for an inhibitory site on the receptor (Karpen & Hess, 1986). As PCP has higher affinity for the inhibitory site than does cocaine (Ulrich et al., 1998), we used PCP to displace the RNA ligands from the receptor. Both cocaine and PCP are non-competitive inhibitors of the nAChR (Karpen & Hess, 1986; Arias et al., 2001; Hess et al., 2003). In the first three cycles, only a nitrocellulose filter-

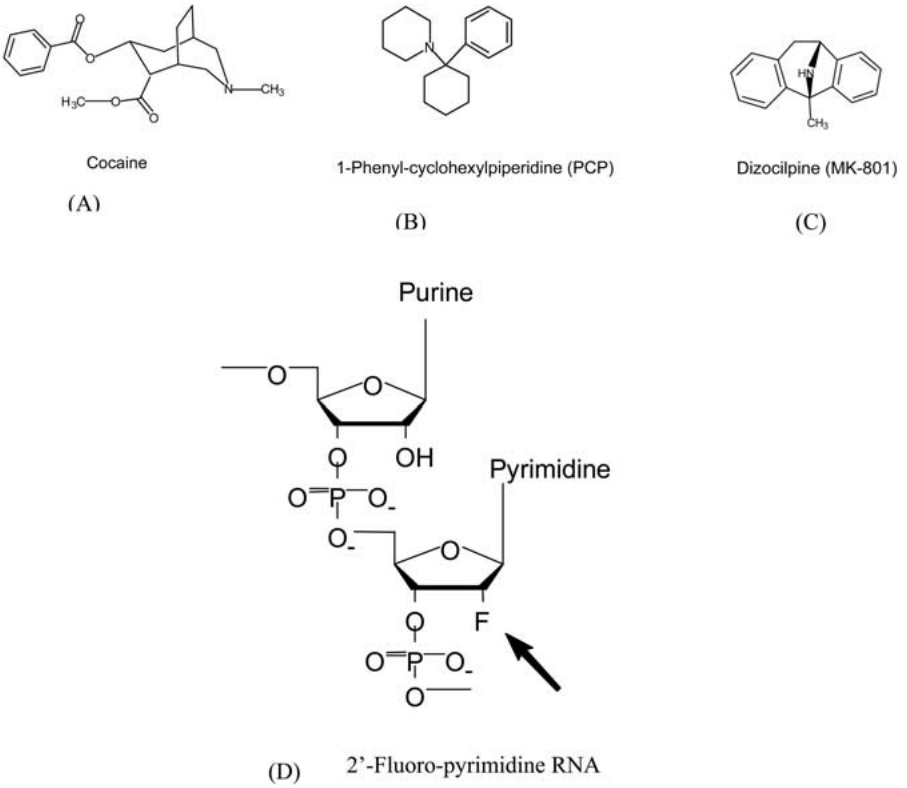


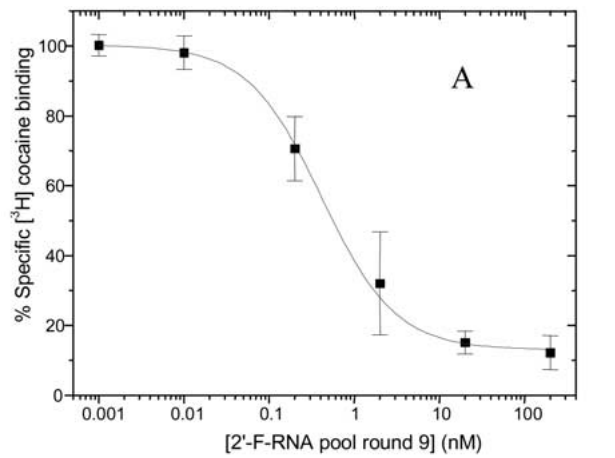
Fig. 2. Structure of (*A*) cocaine, (*B*) PCP, (*C*) MK-801 and (*D*) 2′-Fluoro-pyrimidine RNA.

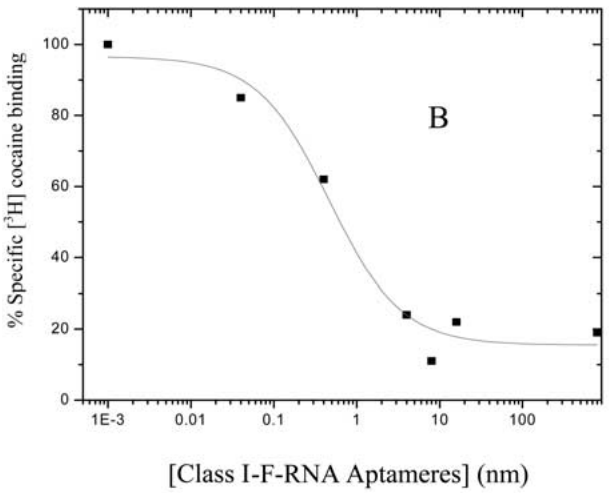
binding selection was carried out to enrich the pool with molecules that bind to the nAChR. In subsequent rounds, the gel-shift procedure (Ulrich et al., 1998) was used before the filter-binding procedure in each selection round to avoid enrichment of RNA molecules that bind to sites other than the selection target.

After SELEX round 9, no further improvement in binding affinity was observed in additional rounds. The binding affinity of the selected aptamer pool was measured by determining the IC_{50} value (McPherson, 1985), which is the concentration at which an inhibitor displaces 50% of a radioactive ligand from the protein. We determined the displacement of [^{3}H]-cocaine from the membrane-bound *T. californica* receptor protein by increasing concentrations of a non-radioactive RNA pool. A mixture of Class I aptamers (numbers 46, 49, 53, and 56), and a mixture of Class II aptamers (numbers 5, 18, 48, and 51) were used. [^{3}H]-cocaine (50 Ci/mmol) was used in the binding experiments. The final concentration of [^{3}H]-cocaine was 100 nM. Specific [^{3}H]-cocaine binding was defined as the difference between binding in the absence and presence of 100 μM unlabeled cocaine and was 75 % of the total binding at 100 μM [^{3}H] cocaine. The IC_{50} values for the RNA pool round 9, Class I aptamers and Class II aptamers are shown in Fig. 3. The IC_{50} of round 9 was determined to be 0.41 ± 0.26 nM (Fig. 3*A*); the IC_{50} of Class I and Class II aptamers are 0.47 ± 0.17 nM (Fig. 3*B*) and 4.1 ± 3.0 nM (Fig. 3*C*), respectively. The displacement of [α-^{32}P]-labeled RNA from the round-9 pool, using cocaine as a competitor, gave an IC_{50} value for cocaine of about 50 μM (*data not shown*). The binding affinities of the stable aptamers presented in this paper are comparable to those obtained previously using unmodified RNA (Ulrich et al., 1998).

The pool from the final SELEX round 9 was cloned into bacterial plasmids and the respective inserts were identified by DNA sequencing. Based on their sequences, 28 individual aptamers were obtained from 70 clones. These 28 aptamers were divided into two classes, based on a consensus sequence (GAAAG or GUUAAU) in the aptamer primary structure. The consensus sequences were identified by aligning the random sequences of the selected aptamers and then determining sequences that were conserved. Structure prediction based on free energy minimization using the MFOLD program (available at http://bioinfo.math.rpi.edu/~mfold/rna/forml.cgi) (Zuker, 1999) revealed that all the aptamers that share this consensus region incorporate the GAAAG or GUUAAU in a stem-loop structure (Fig. 4). The identified consensus regions correlated with aptamer activity, as all aptamers possessing these consensus sequences bound with high affinity (nanomolar to picomolar) to the nAChR.

A subsequent classification was based on the ability of the aptamers to either inhibit the nAChR on BC_3H_1 cells or protect the receptor from inhibition by cocaine as measured by using the cell-flow technique (Table 1*A* and 1*B*). The selected aptamers





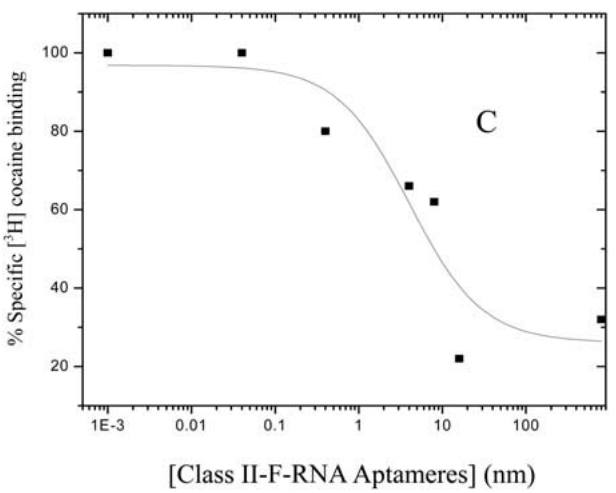


Fig. 3. Displacement by selected 2′-F-RNA pool round 9, and Class I and Class II aptamers of 100 nM [^{3}H] cocaine binding to *T. californica* membrane fragments containing 100 nM nAChR. The IC_{50}, that is the concentration at which an inhibitor displaces 50 % of a radioactive ligand from a protein, can be evaluated from the data presented. *See* the Material and Methods section for details. Data points are the mean ± SD of three experiments. Seventy-five percent of the total binding of 100 nM [^{3}H] cocaine was displaced by the unlabeled cocaine and this 75 percent value was taken as 100 percent specific binding. The binding curve was fitted by the equation $Y = B_{min} + (B_{max} - B_{min})/(1 + [RNA]/IC_{50})$ (McPherson, 1985). Y is the total binding in the presence of various concentrations of the 2′-F-RNA pool, B_{max} is the maximum amount of [^{3}H] cocaine bound, and B_{min} is the amount of [^{3}H] cocaine unspecifically bound to the *T. californica* membranes that cannot be displaced by an excess of RNA aptamers competing for the cocaine-binding site. The IC_{50} value of the 2′-F-RNA pool round 9 was determined to be 0.41 ± 0.26 nM. (*B*) The IC_{50} value of a mixture of Class I aptamers 46,49,53, and 56 was determined to be 0.47 ± 0.17 nM. (*C*) The IC_{50} value of a mixture of Class II aptamers 5, 18, 48, and 54 is 4.1 ± 3.0 nM.

were biologically active when tested for their effect on the fetal muscle-type nAChR in BC_3H_1 cells, but had lower binding affinities than for the *T. californica* nAChR, which was used as the selection target.

Class I-F, Class I-F′ and Class II-F aptamers exert different effects on the nAChR. Class I-F aptamers, at 0.5 μM, inhibit the nAChR (Table 1*A*). The inhibition of the nAChR by Class I-F aptamer 13 is shown in Fig. 5*A* and 5*B*. At the same concentration Class I-F′ and Class II-F aptamers did not inhibit the nAChR. Inhibition of the nAChR by Class I-F′ aptamers appeared at a concentration of 2 μM. Class II-F aptamers up to 2 μM (Table 1*B*) did not show any inhibition of the nAChR. The Class II-F aptamers were further tested for their ability to alleviate receptor inhibition by cocaine.

The results suggest that the aptamer consensus sequence, and the size of the loop structures in which the consensus sequence lies, is related to the biological activity of the aptamers. The structures of Class I-F aptamer 13 and Class II-F aptamer 48 are shown in Fig. 4. Noticeably, both consensus sequences, GUUAAU and GAAAG, are involved in the formation of loops (of 4–6 nucleotides or >6 nucleotides, respectively). Sixteen aptamers in Class I-F and Class I-F′, of the 21 aptamers sharing the consensus sequence GAAAG, have this sequence included in a stem-loop structure larger than six nucleotides (Table 1*A* and 1*B*). Six of the seven Class II-F aptamers sharing the consensus sequence GUUAAU use this motif to form a loop structure containing four nucleotides.

A previous report (Ulrich et al, 1998) of the secondary structure for the nonfluorinated aptamers indicates that Class I molecules that inhibit the receptor and have a loop >6 nucleotides that include UUCGC are potent inhibitors of nAChR activity, and Class II aptamers with a 4-nucleotide loop (AACA) compete with nAChR inhibitors and alleviate the inhibition of the receptor (Hess et al., 2000). Interestingly, although the consensus sequences of the two classes of 2F′-modified aptamers are different from those of the aptamers in which the nucleotides

Table 1. (*A*) Consensus sequences and affinities of Class I-F′ and Class I-F′ (GAAAG) 2′-F-pyrimidine RNA aptamers

Random region sequences (5′-3′)				
Name	Sequence of Class I-F aptamer (active at 0.5 μM concentration)	Number of possible secondary structures[a]	Inhibition of [^{3}H] cocaine binding by aptamers[b]	Numbers of nucleotides forming loop-structure[c]
S1	C**GAACG**UGGACGAAGGGCGGUUUGUGAGUGCUUA	2	1.7	9
S13	CUGACUGCGUCUCUAUAUGACAUAGGCGAUGA**GAAAG**CAGA	1	2.3	10[d]
S14	GGAACAGACGUCAUCUGUG**GCACG**UCCGCUGCUAGCAGAGA	1	1.9	7
S21	GACACAAGCUGGACCACGUCAAGCGUUUUGU**GAAAG**CAGGU	4	1.6	14[d]
S24	UGGCAUCUUGUGCAUGACAACAGAGGGU**GAAAC**CAACGGGU	2	2.5	8
S29	AAACUUGCCUUGGUUUAUAACGUAACAAUACA**GAACG**A	2	1.6	11
S43	AGAAUCUAAGACGU**GAAAA**UGGUAAGACAUUCUCUACC	4	2.7	6
S44	AGGUGUGCGCAGACGAAUAGGGUUGUGC**GAAAG**UCUAGCA	4	2.6	7
S45	UUUAGAGUU**GAAAU**GCGUAAUGGUUAAAUGAUCCAUUCUG	1	1.9	6[d]
S46	AACAAUGCGAGGGU**AAAAG**CAUGUUCUAACCAGGGAGGGA	3	1.4	10[d]
S47	AU**GGAAG**CCCUUGAUUCUACGGAUCUAGCGAGAUUU	2	2.4	4[d]
S49	CCUGUAAGGGCGAAACUAAGCGA**GAAAU**CAUUAGGAUGA	2	1.0	5
S50	CUCAAUGCAUACGCUGGUCAACG**GGACG**AUUAGUGACAAGGCCGC	2	2.2	8[d]
S53	AAUAAGUG**GCAAG**UAGCCUAGAGAUUAGAAGACCUCAAC	1	2.2	7
S56	AAUUGACGAGCUGGUGGGA**GAUAG**UCUCAGGUAUCUUGUGC	5	2.0	5
S59	GGUG**GACAG**UAACUCCUUAGAUGCGGUAGAUUCGUAGC	2	28	7
S62	UACGCGCUUAUGA**UAAAG**GGUUAGAAGGACGAGCGUCGCA	4	2.2	6
Class I-F′(2 μM)				
S15	CACAUGCAGAGUAGU**GUAAG**GUAACACCCAGGUUUUUUG	3	2.6	6
S17	CCGGGGC**GCAGG**UGUCCCUGACGAUGAUCAAUUUCGGGUGA	3	1.8	7[d]
S28	GACGCCUUUAU**GAAUG**ACCAGGGAAGUUGUCAGAAGAGG	6	2.3	12
S54	GUCACUUUCU**GAAUG**GGAGAUAUCUUCGAUAUGGUAAU	2	1.7	9
Consensus	**G A A A G**	Total of 21 Class I-F and		
Frequency	**19 15 19 14 17**	Class I-F′ aptamers		

Table 1. (*B*) Consensus sequences and affinities of Class II-F (GUUAAU) 2′-F-pyrimidine RNA aptamers

Random region sequences (5′-3′)				
Name	Sequence of Class I-F aptamer (active at 0.5 μM concentration)	Number of possible secondary structures[a]	Inhibition of [^{3}H] cocaine binding by aptamers[b]	Numbers of nucleotides forming loop-structure[c]
S5	GAAGGCGAAAGGCACAAAGAUCUGAUGAA**GUUAAU**GGAUCA	3	2.3	4
S18	**GUUAAU**CGCUGAAUAUUCGAAGUGCUUUCCGUGAU	1	2.2	4[d]
S20	UGGGCUUAGGUGUUAAGUCGAUGACU**GUUCAU**UCUCGGUA	4	1.3	3
S25	ACGUGAGCGAGCAAUAAAAGUCCCCUGGGGCGGA**GUUAAA**	5	1.3	4[d]
S48	GGGAGAGUCUACGGAUCCUAGAAAAAGCAGGAC**GUUAUU**	4	3.3	4
S51	CAAAGGGGAGCCACGGGGCGACG**UGUAAU**CCUCUAUUCAGCA	1	2.5	4[d]
S57	AAUGAAGGCAAUUCUUUAAC**GUUAAU**AGGAAGGGGGUAAA	3	1.4	4
Consensus	**G U U A A U**			
Frequency	**6 6 7 6 6 6**	Total of 7 Class II-F aptamers		

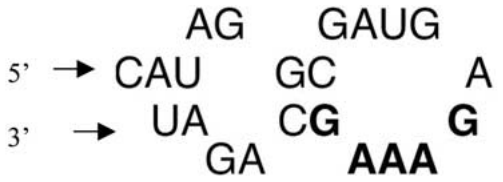
Primary structures of two classes of 2′-fluoro-RNA aptamers based on the consensus sequence and biological activity. Inhibition by aptamers of the nAChR expressed in BC_3H_1 cells was measured by using the cell-flow technique as described in the Materials and Methods section. One hundred μM carbamoylcholine and 0.5 μM aptamer were used in all the aptamer measurements, and aptamers that inhibited under these conditions were assigned to Class I-F. The aptamers that did not inhibit at 0.5 μM were further tested at a concentration of 2 μM. Aptamers that inhibited at this concentration were assigned to Class I-F′. Aptamers that did not inhibit the nAChR were assigned to Class II-F. To determine the ability of aptamers that did not inhibit the nAChR to displace cocaine, 100 nM [^{3}H] cocaine was used in the filter-binding assay (Ulrich et al., 1998). Five-hundred nM aptamer was used to displace [^{3}H] cocaine. In all cases the general sequences of the RNA aptamers are:

5′-GGGAGACAAGAAUAAACGCUCAA [40 N variable region] UUCGACAGGAGGCUCACAACAGGC-3′.

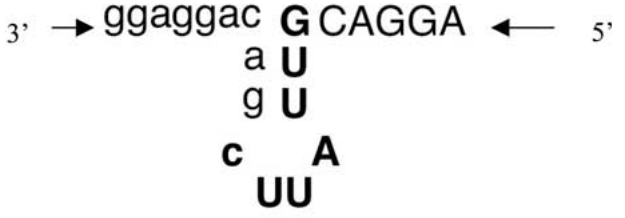
[a]Predicted by the MFOLD program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) (Zuker, 1999).

[b]The inhibition by aptamers of [^{3}H] cocaine binding was expressed as the ratio of [^{3}H] cocaine binding to 500 nM (final concentration) nAChR in the absence and presence of 500 nM (Class I-F and Class II-F) or 2 μM (Class I-F′) aptamers.

[c]In this column the numbers of nucleotides forming the loop structure are shown. These loop structures are formed by the consensus

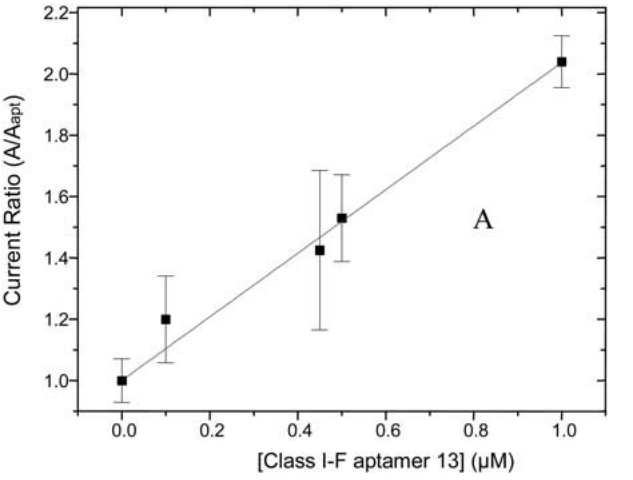


Class I-F aptamer 13



Class II-F aptamer 48

Fig. 4. Secondary structures of Class I-F aptamers and Class II-F aptamers containing the consensus sequences. The secondary structures of the aptamers were obtained by using the MFOLD software (http ://bioinfo.math.rpi.edu/~infold/rna/form 1. cgi). The variable region is in upper-case letters, the constant region in lower-case letters, and consensus sequences are in bold font. Class I-F aptamer 13 with a GAAAG consensus sequence forms a larger loop of 11 nucleotides. Class II-F aptamer 48 forms a smaller loop containing the consensus sequence GUUAUU.



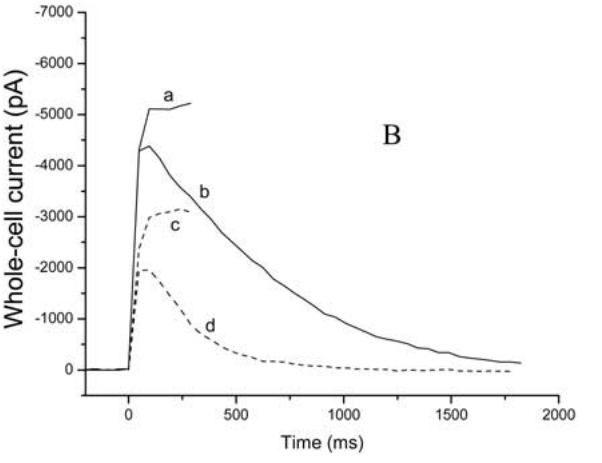


Fig. 5. Inhibition of the function of muscle type-nAChR in BC_3H_1 muscle cells by Class I-F aptamer 13, as measured by using the cell-flow technique. (*A*) The whole-cell current was measured by using the cell-flow technique (Udgaonkar & Hess, 1987) and a constant concentration (100 μM) of carbamoylcholine. The cells were pre-equilibrated with Class I-F aptamer 13 for 2 s. The line was fitted by the equation $A/A_{apt} = 1 + [aptamer]/K_{apt}$ (Niu & Hess, 1993); A and A_{apt} represent the current amplitude corrected for desensitization (Udgaonkar & Hess, 1987) in the absence and presence of an increasing concentration of Class I-F aptamer 13, respectively. K_{apt} is the apparent inhibition constant of the aptamer. From the data shown, $K_{apt} = 0.97 \pm 0.2$ μM for this aptamer. (*B*) The whole-cell current-recording technique was used in combination with the cell-flow technique at a membrane potential of −60 mV and 22 °C in BC_3H_1 buffer, pH 7.4. The lines parallel to the abscissa represent currents corrected for receptor desensitization (Udgaonkar & Hess, 1987). Current traces designated as *a* and *b* were obtained in the presence of 100 μM carbamoylcholine, and currents *c* and *d* were obtained in the presence of 100 μM carbamoylcholine and 1 μM Class I-F aptamer 13. Curves labeled *a* and *c* are the currents corrected for receptor desensitization (Udgaonkar & Hess, 1987) and those labeled *b* and *d* are the observed currents.

are not fluorinated, the number of nucleotides forming a stem-loop structure of the two groups with either inhibition (Class I, Class I-F, and Class I-F′) or alleviation (Class II and Class II-F) characteristics is surprisingly similar; aptamers with more than 6 nucleotides in a stem loop inhibit, and those with 4 nucleotides in the stem loop alleviate, regardless of whether or not they contain fluorinated nucleotides. A previous study (Ulrich et al., 1998) indicated a relationship between aptamer activity and consensus sequence. The results presented here suggest that aptamer activity is also related to the number of nucleotides in the stem loop.

Class II-F aptamers alleviate inhibition of the nAChR by cocaine or MK-801. MK-801 (Fig. 2*C*), a secondary amine, has anticonvulsant properties and alleviates the effects of cocaine intoxication (Ramoa et al., 1990; Rockhold et al., 1992). It also inhibits the nAChR (Grewer & Hess, 1999; Hess et al., 2000). In our cell-flow measurements, MK-801 and cocaine were chosen as inhibitors for determining the ability of the aptamers to prevent receptor inhibition. The values of the observed dissociation constants, K_{apt} (obs), for the alleviation by Class II-F aptamer 51 (Fig. 6*A*) or Class II-F aptamer 48 (Fig. 6*B*) and by a mixture of Class II-F aptamers (Fig. 6*C*) are in the picomolar range. The measurements showed that Class II-F aptamers can displace MK-801 and alleviate receptor inhibition. Class II-F aptamer 5 also alleviates cocaine inhibition

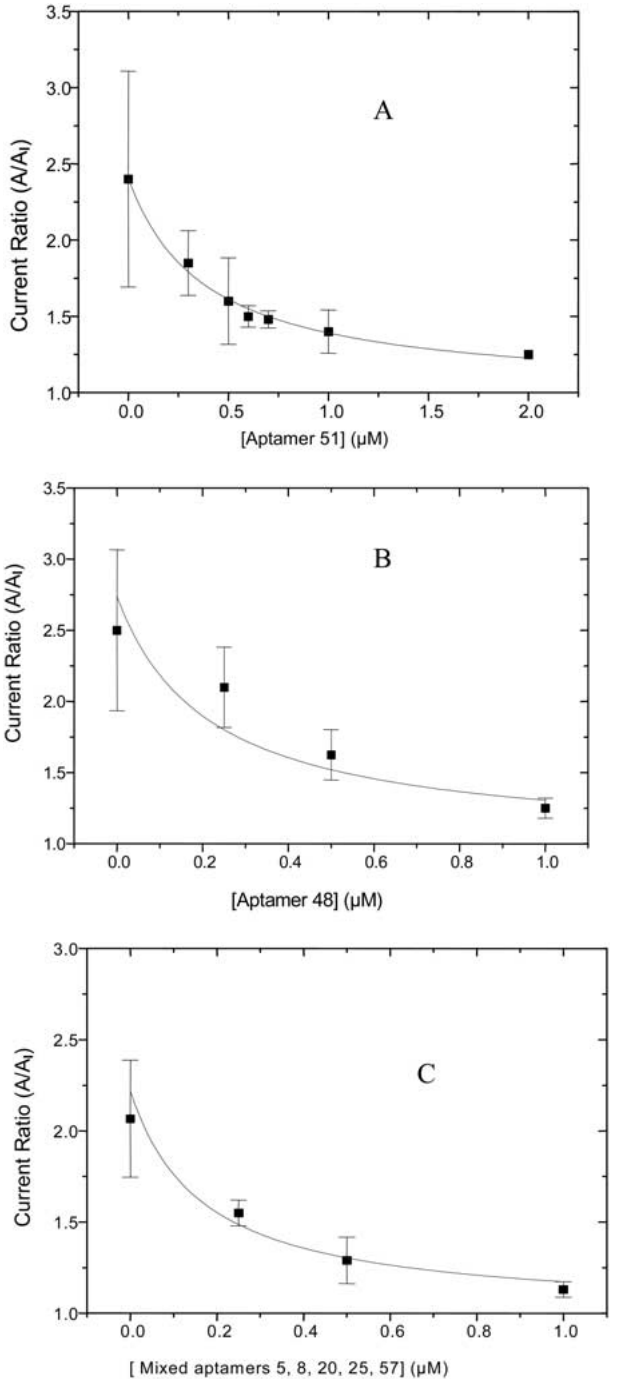


Fig. 6. Alleviation by Class II-F aptamers of MK-801 inhibition of the nAChR. The recorded whole-cell currents were corrected for receptor desensitization by using the cell-flow technique (Udgaonkar & Hess, 1987). 100 μM carbamoylcholine was the activating ligand and 500 μM MK-801 was the inhibitor. The cells were pre-equilibrated with from 0.1 to 2 μM Class II-F aptamers for 4 at 25 °C, pH 7.4; the experiment was carried out at a membrane potential of –60 mV in BC_3H_1 buffer. Each data point is the mean ± SD of three experiments. K_I (*obs*) and K_A (*obs*) represent observed dissociation constants of the inhibitor or the aptamers from the receptor, respectively. (*A*) Class II-F aptamer 51, K_I (*obs*) = 340 ± 150 μM and K_A (*obs*) = 0.3 ± 0.2 μM. (*B*) Class II-F aptamer 48, K_I (*obs*) = 287.6 ± 90.0 μM and K_A (*obs*) = 0.21 ± 0.10 μM. (*C*) Mixed aptamers (Class II-F 5, 18, 20, 25,

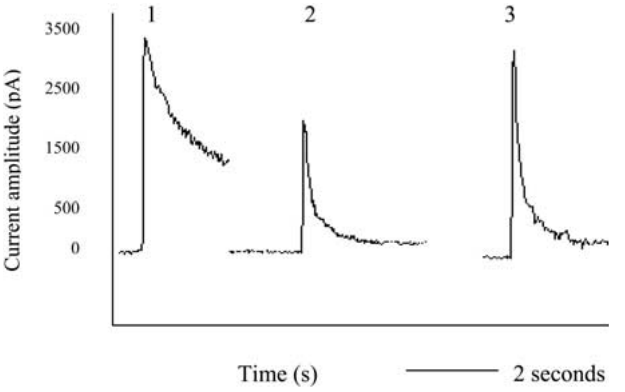


Fig. 7. Alleviation by Class II-F aptamer 5 of cocaine inhibition of the nAChR. For alleviation of cocaine inhibition in the presence of 100 μM carbamoylcholine, the current amplitude was obtained in the absence and presence of 200 μM cocaine and in the presence of 2 μM Class II-F aptamer 5 at a membrane potential of –60 mV, pH 7.4, 22°C in BC_3H_1 buffer. Peak *1*: 100 μM carbamoylcholine. Peak *2*: 100 μM carbamoylcholine and 200 μM cocaine. Peak *3*: 100 μM carbamoylcholine, 200 μM cocaine and 2 μM Class II-F aptamer 5. The figure presents representative current traces; data was normally obtained by using at least two cells for each experiment.

of the nAChR (Fig. 7). Although Class I and Class II aptamers have different functions with respect to the nAChR, both were selected for the cocaine-binding site of the nAChR. Displacement of [^{3}H] cocaine by Class I and Class II aptamers indicates that both classes of aptamers bind to the cocaine-binding site. Our previous results suggested that the nAChR has a cocaine-binding site with different binding affinities on the open- and closed-channel forms of the receptor. This explains why Class I aptamers inhibit the receptor and Class II aptamers displace cocaine or MK-801 from the receptor without inhibition, each class having a different consensus sequence.

Notably, the K_d values obtained using the cell-flow technique are different from the IC_{50} values obtained from binding experiments. One reason is that the aptamers were selected against the *T. californica* nAChR, which is structurally similar but not identical [78 % similarity of α subunits on the protein level determined by using the Clustal software (Pearson & Lipman, 1988)] with the fetal muscle type nAChR in BC_3H_1 mouse cells used for cell-flow measurements. This may explain why the selected aptamers have a dissociation constant in the low nanomolar region when measured with the *T. californica* receptor, but are active on the muscle-type mouse nAChR only at upper nanomolar concentrations. These observations are in accordance with the findings of Ulrich and colleagues (Ulrich et al., 1998) who selected unmodified RNA aptamers against the *T. californica* nAChR and characterized their effects on the nAChR on BC_3H_1 cells.

The stability of the Class II-F aptamer 51 and of an aptamer with the same RNA sequence but not

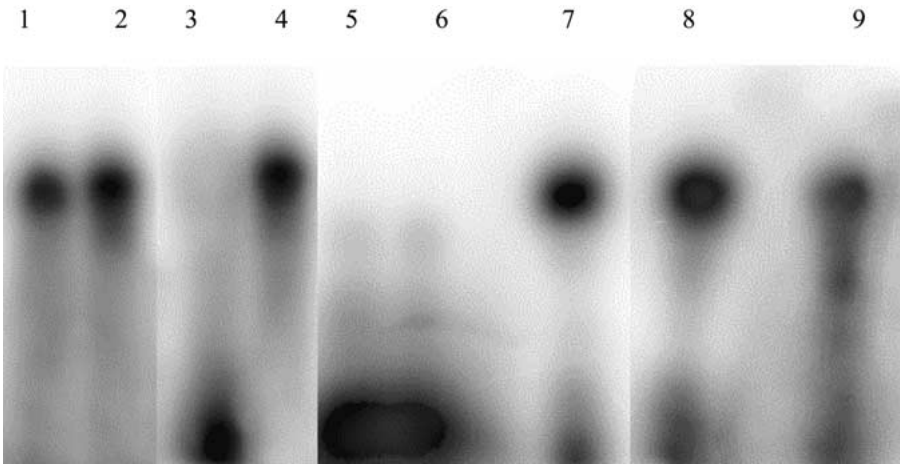


Fig. 8. Comparison of the stability of Class II-F aptamer 51 and the unmodified aptamer 51, with identical sequences, in human serum and urine. Lane *1* (Class II-F aptamer 51) and lane *3* (unmodified aptamer 51): incubated in 90% human serum for 4 h. Lane *2* (Class II-F aptamer 51) and lane *4* (unmodified aptamer 51) are the controls (without serum). Lane *5* (unmodified aptamer 51 with 2U/μl anti-RNase), lane *6* (unmodified aptamer 51 without anti-RNase) and lane *9* (Class II-F aptamer 51 without anti-RNase): incubated in 90% human urine for 4 h; lane *7* (unmodified aptamer 51) and lane *8* (Class II-F aptamer 51) are the controls (without urine). *See* Materials and Methods section for other experimental details.

modified with fluorine were measured. The stability of Class II aptamer 51 in 90 % human serum and in 90 % human urine was compared by running radiolabeled RNA, with [α^{32}P]-ATP incorporated during transcription, on an 8 % denaturing polyacrylamide gel (Fig. 8). The tests indicated that there was no obvious degradation of the Class II-F aptamer, whereas the unmodified Class II-F RNA aptamer was completely degraded in both serum and urine after incubation for 4 h. Further time-dependent experiments using the same techniques described above showed that about half of the Class II-F aptamer 51 was still full-length after a 48 h incubation, whereas unmodified RNA in both serum and urine had completely degraded in less than 20 min. Pagratis and colleagues (Pagratis et al., 1997) indicated that the half-life of unmodified RNA in serum is <8 seconds. The result demonstrated that Class II-F RNA is significantly more stable in both biological fluids, which are rich in degradative enzymes. It needs to be emphasized that the stabilization of cocaine-displacing aptamers against nuclease activity is an important step towards the use of these molecules in characterizing the aptamer-binding sites of the receptor that are embedded in cell membranes and in diagnosis and therapy. The aptamers can be studied together with cocaine in animal models, and cellular physiology and animal behavior can be studied following reversion of cocaine inhibition of the nAChR. It is worthwhile to note that the first aptamers directed against growth factors, such as the vascular epidermal growth factor (VEGF), were made using unmodified nucleotides (Jellinek, 1994) as a proof of the principle that an aptamer recognizes its target with the same specificity as an antibody. Based on these findings, nuclease-resistant aptamers were synthesized that target this class of proteins (Ruckman et al. 1998), resulting in the discovery of the anti-VEGF aptamer (NX-1838) (Sun, 2002). This is the first aptamer approved in clinical tests for treatment of a disease (Eyetech, 2003). Similarly, identification of cocaine-displacing aptamers may also open new avenues for the treatment of cocaine intoxication.

In addition, we have demonstrated that the SELEX method (Ellington & Szostak, 1990; Tuerk & Gold, 1990) is suitable for finding ligands that bind to membrane-bound proteins of pharmacological or therapeutic importance. In principle, for almost every target protein, a stable aptamer acting as an agonist or antagonist should be obtainable, thus opening new perspectives for diagnosis and therapy.

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