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Water soluble RNA based antagonist of AMPA receptors

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Abstract

α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are one of the important receptor classes involved in glutamate-mediated excitotoxicity. Although small molecule antagonists of this receptor have been shown to have neuroprotective properties, their low solubilities pose severe side effects in clinical trials. Here we have used the SELEX method to obtain water-soluble nuclease-resistant RNA ligands that bind to the agonist binding site of AMPA receptors. Using whole-cell current recordings, we have characterized the functional consequences of a representative aptamer from this class and show that it is a competitive antagonist of AMPA receptors and in the concentration range where it acts as an inhibitor of the AMPA receptor the RNA has no effect on the GluR6 homomeric kainate receptors. Additionally, using a fluorescence resonance energy transfer (FRET) probe, we show that this RNA ligand stabilizes the open cleft conformation of the ligand binding domain, consistent with the known structures of small antagonist-bound states of the soluble domain of this protein. Finally, using rat primary cortical neurons, we show that this RNA ligand significantly reduces neurotoxicity associated with oxygen glucose deprivation. The water-soluble and antagonistic properties of this aptamer coupled to its neuroprotective properties make it an excellent candidate for potential use in diseases or pathological conditions involving glutamate-mediated excitotoxicity.

Keywords

RNA aptamers; AMPA receptors; ALS; excitotoxicity; fluorescence; Neuroprotective

Introduction

Antagonists of ionotropic glutamate receptors are considered to have clinical potential in many neurological disorders involving neurodegeneration ranging from acute events such as stroke or epilepsy to chronic diseases such as Parkinson's disease and amyotrophic lateral sclerosis (Kawahara and Kwak, 2005; Kwak and Weiss, 2006; Sun et al., 2006). Recent focus has been on the antagonists of the AMPA subtype of the glutamate receptors since they do not produce

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the adverse psychotomimetic and cardiovascular effects as observed for antagonists of the N-methyl D-aspartic acid (NMDA) receptor subtype (Planells-Cases et al., 2006). Several series of AMPA receptor antagonists are reported in the literature including AMPA-derived antagonists as well as the quinoxalinedione derivatives (Madsen et al., 2001). Antagonists of AMPA receptors have been shown to be consistently effective in reducing neuronal death in various experimental models (Canton et al., 2001; Van Damme et al., 2003; Van den Bosch et al., 2002). However, their full potential has not been realized in clinical applications due to their severe side effects that can be mainly attributed to their insolubility (Herrling, 1997; Sheardown et al., 1990). To overcome the limitations of existing drugs, here we report a new class of high affinity RNA aptamers that are water-soluble inhibitors of AMPA receptors.

We have obtained these high affinity RNA aptamers through an *in vitro* selection process termed SELEX (systematic evolution of ligands by exponential enrichment), in which RNA aptamers are selected from a library of random RNA sequences by repetitive binding of the RNA molecules to target molecules (Tuerk and Gold, 1990; Ulrich, 2005a). Furthermore, we have generated nuclease-resistant RNA ligands by the incorporation of 2'-fluoro pyrimidine into the RNA, since these work effectively as drugs *in vivo* in animal models (Lee et al., 2005; Ruckman et al., 1998) and are promising candidates for use in pharmaceutical and therapeutic applications (Ulrich, 2006).

Furthermore, we also show that the SELEX methodology can be used with a minimal construct that exhibits the required drug binding properties and does not require the use of the full length receptor/protein. Specifically, for evolving RNA aptamers that bind to AMPA receptors, we have used the isolated ligand binding domain of the GluR2 subunit of this subtype (GluR2-S1S2) that has previously been shown to be an excellent model for this domain in the full receptor (Armstrong and Gouaux, 2000; Deming et al., 2003; Du et al., 2005). The purified GluR2-S1S2 protein can be expressed in large quantities, thus making the SELEX procedure relatively easy as opposed to selecting RNA aptamers using membrane preparations. The RNA aptamers thus evolved have been studied using electrophysiological and fluorescence resonance energy transfer measurements to characterize the functional and structural consequences of their binding to AMPA receptors, respectively. Additionally, we also present preliminary studies showing the neuroprotective properties of the evolved aptamer using the oxygen-glucose deprivation model, thus establishing the potential use of these ligands as pharmaceutical agents for neuroprotection.

Materials and Methods

Chemicals

Glutamate was purchased from Sigma (St. Louis, MO), [α - 32 P]-ATP (30Ci/mmol) and L-[γ - 3 H]-glutamic acid (49.0Ci/mmol) were purchased from GE Healthcare Life Sciences (Piscataway, NJ). 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), and [3 H]-AMPA (45.5 Ci/mmol) and [3 H]-kainate (47 Ci/mmol) were purchased from PerkinElmer (Shelton, CT).

Expression and purification of GluR2-S1S2

The plasmid pET22b(+) GluR2S1S2J, used to express GluR2-S1S2, was provided by Dr. Eric Gouaux (Oregon Health Science University, OR). The wild type GluR2-S1S2 protein used for the SELEX experiments and the T394C-S652C mutant of GluR2-S1S2 used for the FRET measurements were expressed and purified as described previously (Armstrong et al., 2003; Ramanoudjame et al., 2006). The buffer used for the SELEX and FRET experiments contained 25 mM HEPES, 145 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl₂ and 1.7 mM MgCl₂, at pH 7.4.

Expression of full length receptor

The cDNAs for GluR2 flip and GluR4 flip were provided by Dr. Peter Seeburg (Max Planck Institute, Germany), GluR1 flip and GluR3 flip forms were provided by Dr. Christian Rosenmund (Baylor College of Medicine, TX), and GluR6 was provided by Dr. Kathryn Partin (Colorado State University, CO). The receptors were expressed in HEK 293 cells using either Eugene 6 (Roche, IN) or Lipofectamine transfection reagents (Invitrogen, Carlsbad, CA). Electrophysiological measurements were performed two to three days after transfection. For the radioactive ligand binding experiments, membranes were prepared with transfected cells using the protocol described by Ulrich et al. (Ulrich, 2005b).

Preparation of 2'-F-pyrimidine RNA pool

The PAGE-purified 108-nt single-stranded DNA library and two HPLC-purified primers were obtained from Sigma-Genosys (The Woodlands, TX). The single-stranded DNA template contained a 40-nt central randomized region flanked by two constant regions (5'-ACCGAGTCCAGAAGCTTGTAGTACT-N40-GCCTAGATGGCAGTTGAATTCTCCCTATAGTGAGTCGTATTAC-3'). The two primers used for the amplification of double-stranded DNA were: 5' GTAATACGACTCACTATAGGGAGAATTCAACTGCCATCTA-3' (P-40), and 5' ACCGAGTCCAGAAGCTTGTAGT-3' (P-22) (Ulrich, 2005b; Ulrich et al., 1998).

The initial nuclease resistant 2'-F-pyrimidine RNA pool (RNA pool 0) was obtained by *in vitro* transcription of the 108-nt DNA pool using T7 RNA polymerase (Ambion, Austin, TX) and 2'-fluoro-modified pyrimidine nucleotide triphosphates (Ulrich et al., 1998). The template DNA was removed using RNase-free DNase I (2 U/μl) (Ambion). The RNA thus obtained was heat denatured at 65°C and renatured at room temperature to allow the formation of secondary structures and its integrity verified using a 10% denaturing polyacrylamide gel.

Binding Analysis of selected RNA to GluR2-S1S2

Displacement binding studies were performed using [³²P]-labeled RNA that was generated by transcribing 3-5 μg of the DNA template using 0.5 mM GTP, 2.5 μM ATP, 1.5 mM 2'-F-pyrimidines, and 50 μCi of [α-³²P]ATP. For the displacement studies, a 32 nM [³²P]-RNA pool was incubated with 1.2 μM GluR2-S1S2 (2 μg) in the presence of 0.3 μg/μl t-RNA with increasing concentrations of glutamate in 50 μl of selection buffer. The reaction mixtures were separated by filtration through a nitrocellulose filter, and the radioactivity retained on the filters was counted using a liquid scintillation counter. From the displacement curves, the IC₅₀ value (concentration of glutamate required to displace 50% of the RNA from GluR2-S1S2) was calculated by fitting the data to equation 1:

$$y = B_{\min} + \frac{B_{\max} - B_{\min}}{1 + \frac{[Glutamate]}{IC_{50}}} \quad \text{Eq. 1}$$

where $B_{\max}$ and $B_{\min}$ are the maximum and minimum radioactivity counts. Using the IC₅₀ value, the approximate dissociation constant, K_R , for the RNA was established using the Cheng-Prusoff equation (eq.2):

$$K_d = \frac{IC_{50}}{1 + \frac{[^{32}P-RNA]}{K_R}} \quad \text{Eq. 2}$$

where K_d is the dissociation constant for glutamate, and [³²P-RNA] is the concentration of the radio-labeled RNA.

Binding assay of RNA aptamers for Kainate and AMPA full length receptors

The binding affinity of the individual RNA aptamer for the full length receptors was determined by saturation binding analysis using HEK 293 cell membranes transiently expressing the receptors. Constant amounts of membrane protein (12 µg) were incubated with increasing concentrations of the [³²P]-labeled individual RNA aptamer in the presence of 0.3 µg/µl yeast t-RNA in 50 µl of selection buffer for 60 min at room temperature. Bound and free radioligands were separated by rapid-filtration using Whatman nitrocellulose filters. Radioactivity was determined by counting with a liquid scintillation counter. Non-specific binding of [³²P]-RNA was determined by including 10 mM glutamate in the binding assay. The functionality of the receptors in the membrane preparations was established by AMPA for the AMPA subtype and by kainate for the GluR6 receptors using [³H]-AMPA and [³H]-kainate. The K_d values for the ligands were determined by non-linear fitting of the binding data using equation 3:

$$y = \frac{x \times B_{\max}}{x + K_d} \quad \text{Eq. 3}$$

where y is the total binding of the radioactive ligand, $B_{\max}$ is the maximum amount of bound radioactive ligand, and x the concentration of free ligand.

Characterization of the function of individual RNA ligands by whole cell current-recording measurement

Whole-cell current-recordings were used to evaluate the effect of the individual RNA aptamer on the function of glutamate receptors. The transfected HEK 293 cells were voltage-clamped at a holding potential of -60 mV, and solutions were applied by using a homemade U-tube mixing device that had a 100 µm aperture. The electrode solution, for the electrophysiological measurements, contained 140 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, 2 mM Na₂ATP, and 10 mM HEPES (pH 7.4); the extracellular bath solution contained 145 mM NaCl, 1.8 mM MgCl₂, 1 mM CaCl₂, 3 mM KCl, 10 mM glucose, and 10 mM HEPES (pH 7.4). Currents were amplified with an Axon 200B amplifier (Molecular Devices) and low-pass filtered at 1 kHz. The filtered signal was digitized by using a Labmaster DMA digitizing board controlled by pClamp (Molecular Devices, Sunnyvale, CA). All experiments were performed at room temperature.

For investigating the inhibitory properties of RNA aptamers the cells were preincubated for at least 5 seconds prior to the application of solution containing glutamate and the RNA aptamer. This time was sufficient to determine the full extent of inhibition, i.e. preincubation for longer periods of time did not change the extent of inhibition by the RNA.

To determine the EC₅₀ values, the dose response curves were fit to equation 4.

$$y = I_{\min} + \frac{I_{\max}}{1 + \left(\frac{EC_{50}}{x}\right)^n} \quad \text{Eq. 4}$$

Where $I_{\min}$ and $I_{\max}$ are the minimum and maximum normalized currents and n is the Hill coefficient.

Characterization of individual RNA aptamer by fluorescence lifetime measurements

0.1-0.25 µM GluR2-S1S2-T394C-S652C protein in selection buffer containing 1mM glutamate was labeled with maleimide derivatives of terbium chelate (TTHA-Tb, Invitrogen) and fluorescein at a ratio of 1:1. The labeled protein was then thoroughly dialyzed against buffer before performing the fluorescence lifetime measurements. The 1:1 labeling lead to donor only, acceptor only, as well as donor-acceptor tagged protein. Using temporal and

spectral separation we can study the fluorescence lifetimes from donor-acceptor tagged protein as described in detail by Ramanoudjame et al. (Ramanoudjame et al., 2006). The FRET efficiencies which reflect the distance between the donor and acceptor were characterized by the FRET lifetimes and data were analyzed using Origin software (OriginLab, Northampton, MA).

Primary cortical neuron cultures

Primary neuron cultures were prepared from E15 to E16 day rat embryos as described before (Zhao et al., 2006). Briefly, the neocortices were dissected and dissociated by trituration. The dissociated cells were then plated onto poly-L-lysine coated culture plates in Neurobasal medium with B27 at a density of 700/mm² and cultured in a CO₂ incubator (5% CO₂, 21% O₂) at 37.0 ± 0.5 °C. The culture medium was changed every 3 days. After a total of 12 days in culture, the cells formed extensive axonal and dendritic networks and were then used for experiments.

Oxygen-Glucose-Deprivation Injury

The cell injury was induced by subjecting the cultured cells to oxygen-glucose-deprivation (OGD) for 2 h as previously described (Kim et al., 2004). Briefly, the cultures were exchanged into neurobasal medium without glucose (Invitrogen, Carlsbad, CA) and then placed in a gas-tight humidified chamber and flushed with a gas mixture consisting of 5% CO₂/ 95% N₂ for 10 min. The chamber was then closed and placed at 37 °C for 2 hours. At the end of OGD injury, glucose was added into the cultures, and the culture plates were returned to the original culture incubator for re-oxygenation. The cell survival/death was measured 24 h after OGD.

Administration of RNAs

0.02 or 0.1 μM of either individual RNA aptamer or the initial RNA pool (pool 0 prior to SELEX) were added directly to the culture medium 1 h before OGD. The RNA aptamer remained in the media throughout the experiment. The initial RNA pool 0 was used as negative control while the AMPA receptor antagonist CNQX (dissolved in 0.5% DMSO) was included as positive control.

LDH Assay

Assessment of cell injury was performed by measuring the release of lactate dehydrogenase (LDH) into the culture media using an LDH assay kit (Promega, Madison, WI). Briefly, 100 μl of culture media was collected from each culture well and incubated with 100 μl of the LDH assay reagent for 30 minutes in a 96-well plate. The colorimetric value (OD) was determined using an ELISA Reader (Bio-Rad) at 490 nm. The data were expressed as percentage over control.

MTT Assay

The neuronal viability after OGD was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Promega, Madison, WI). Briefly, the soluble tetrazolium was added into the neuron culture media at 20h after the onset of OGD and incubated for 4h. The insoluble formazan that formed in the metabolically viable neurons was dissolved with a mixture of isopropanol/formic acids (95/5), and the optical densities were measured at 545 nm and expressed as mean ± SEM.

FDA-PI Stain

Cell injury was quantitatively assessed by examining nuclear uptake of propidium iodide (PI) and somal loss of fluorescein diacetate (FDA) as was described in Pike et al. (Pike et al.,

1998). The freshly stained cells were viewed under a Zeiss photomicroscope in epifluorescence configuration. Four (2 columns $\times$ 2 rows) of independent fields (each field = $448 \times 335 \mu\text{m}$) from each culture well were captured using a DVC color CCD camera. Digitized images were processed by MetaMorph program (Universal Imaging Corporation). The total number of living and dead cells in each field was counted. The percentage of cell death [(number of average green in control - number of green in each field) / (average number of total red + green in control) $\times$ 100] was expressed as mean $\pm$ SEM.

Statistical Analysis

All data were expressed as mean $\pm$ SEM. Two independent experiments were performed in triplicate (3 culture wells each time). The data were analyzed using Prism and InStat (GraphPad Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by the Newman-Keuls post-test was used to evaluate the difference among the groups. $P \leq 0.05$ was considered as significant.

Results

Isolation of 2'-F-RNA aptamers that bind with high affinity to glutamate-binding sites in AMPA receptors

For the *in vitro* selection, the 2'-F-RNA pool was incubated with GluR2-S1S2 protein, and the bound RNA was separated from the unbound RNA by using a nitrocellulose filter and a 96-well Minifold filtration apparatus. After washing the complexes with buffer, the protein-bound RNA was displaced with $500 \mu\text{M}$ glutamate. The collected supernatant containing the displaced RNA was phenol and chloroform-extracted, and the purified RNA was used as template for reverse-transcription (RT) PCR amplification to obtain the DNA pool for the next SELEX round. Since the RNA aptamers were being selected based on their affinity to the isolated ligand binding domain, a negative selection was performed after two initial SELEX rounds using membranes of non-transfected HEK 293 cells. The RNA molecules that did not bind to the non-transfected HEK 293 cell membranes were then used for subsequent selection rounds towards GluR2-S1S2.

After eight repetitive selection rounds, the affinity of the RNA pool 8 for binding to GluR2-S1S2 was determined using a radioligand-receptor binding assay, as shown in Figure 1. Using equation 1, the concentration of glutamate required to displace 50% of the RNA pool 8 from the ligand-binding sites of GluR2 (IC_{50}) was determined to be $1 \pm 0.2 \mu\text{M}$, and using the Cheng Prusoff equation and K_d value of 500 nM for glutamate (Armstrong and Gouaux, 2000), this IC_{50} value corresponds to a dissociation constant for the RNA pool 8 receptor binding (K_R) of $25 \pm 8 \text{ nM}$. To determine the sequence of the individual aptamer sequences constituting this pool cDNAs were generated and cloned into bacterial plasmids and the respective inserts were sequenced from individual bacterial colonies. Among these sequences a 7-nt consensus motif CCCCCA was found in 14 of 30 identified RNA aptamers (Table 1), three of these 14 aptamers were tested using radioactive binding.

Radioactive binding of RNA aptamers

The binding properties of RNA aptamer #1, #10, and #25, were tested using membranes of HEK 293 cells expressing GluR2-flip receptors (Supplemental figure 1). The saturation binding curves show that aptamers #1, #10, and #25, bind to the receptor with similar K_d values (listed in Table 2A), suggesting that the RNA aptamer binds at the consensus sequence and that a change of one base pair in the consensus sequence does not have a significant effect on the binding affinity.

To further analyze the binding properties of the aptamer, the binding properties of aptamer #25 were tested using different AMPA receptor subtypes and also a kainate receptor (GluR6) which belongs to the non-NMDA subtype of glutamate receptors and is closely related to AMPA receptors (Table 2B, Supplemental Figure 1C and Supplemental Figure 2). The K_d values for aptamer #25 binding to GluR1-flip, GluR3-flip, and GluR4-flip receptors was 190 ± 15 nM, 47 ± 4 nM and 70 ± 15 nM, respectively. These values are similar to the value of 37 ± 5 nM determined for GluR2-flip receptors suggesting that the aptamers are not selective for the GluR2 subtype of the AMPA receptors. However, the binding studies with GluR6 (kainate) receptors, on the other hand, show that RNA aptamer #25 does not exhibit saturation up to 200 nM, indicating a low affinity binding. These data suggest that the RNA aptamer bind with a higher affinity to the AMPA receptors relative to the GluR6 kainate receptor.

Functional consequences of RNA binding

Whole-cell currents were measured using HEK 293 cells expressing the homomeric GluR1-flip, GluR2-flip, GluR3-flip, GluR4-flip and GluR6 receptors to test the functional properties of aptamer #25. No currents were observed upon application of 5 μ M aptamer #25, suggesting that aptamer #25 is not an agonist of the receptor at these concentrations (Figure 2 A). The whole cells currents mediated by 250 μ M glutamate, on the other hand, were inhibited in the presence of 1 μ M aptamer #25 for GluR1-flip, GluR2-flip, GluR3-flip and GluR4-flip receptors (Figure 2B-E and Figure 3), thus indicating that aptamer #25 is an antagonist of the receptor. No significant effect was observed on whole cell currents mediated by 250 μ M glutamate due to the presence of 1 μ M aptamer #25 for GluR6 receptors (Figure 2 F and Figure 3). These results suggest that aptamer #25 at 1 μ M exhibits selective inhibition of AMPA receptors and has no effect on the closely related GluR6 (kainate) receptor. RNA aptamer #10 was also studied to test if this inhibitory property was observed in another aptamer exhibiting the consensus sequence, and a similar inhibitory effect was observed on the GluR2-flip receptor as that due to RNA aptamer #25 binding (Figure 2G and Figure 3).

To examine in more detail the mechanism of inhibition by aptamer #25, dose response curves were obtained for glutamate activation of GluR2-flip receptors (Figure 4). The dose response for whole cell currents mediated by glutamate were shifted to higher concentrations in the presence of 1 μ M RNA aptamer #25, and the EC_{50} increased with higher aptamer concentrations. The increase in the EC_{50} value due to increasing concentrations of the aptamer #25 indicates that aptamer #25 is a competitive inhibitor. Due to the high cost involved in making the RNA, only three concentrations of RNA were probed. However, even with this limited data, the Schild plot (Figure 5) of the EC_{50} values clearly exhibits a linear trend, with a slope of -0.99 , consistent with a competitive inhibitor. The approximate K_d of the aptamer obtained from the x-intercept of the Schild plot is 1.20 ± 0.15 μ M. The extent of inhibition obtained by varying the concentration of aptamer #25 at 250 μ M glutamate is shown in Figure 6. Again due to the cost of making high concentrations of RNA, the complete inhibition curve was not obtained. However, the data allows for an approximate characterization of the IC_{50} value, which corresponds to 1.0 ± 0.1 μ M. This value of IC_{50} is in good agreement with the K_d value of the aptamer, as expected due to the fact that the inhibition curve was obtained at glutamate concentration close to the EC_{50} value of glutamate.

Structural consequences of RNA binding

The FRET lifetimes of a donor (terbium chelate): acceptor (fluorescein) tagged T394C-S652C mutant of GluR2-S1S2 were used as a probe to study the structural consequences of aptamer #25 binding. We have previously demonstrated that the cleft closure conformational change induced by agonist binding is reflected as a decrease in the FRET lifetimes using this probe (Ramanoudjame et al., 2006), consistent with a decrease in the distance between the donor and acceptor fluorophores. This conformational change is thought to be the primary control of

receptor activation. Consistent with this hypothesis, small molecule antagonists have been shown to stabilize the apo-like open cleft conformation of the GluR2-S1S2 protein (Armstrong and Gouaux, 2000).

The FRET lifetimes, as measured by the sensitized emission for the modified GluR2-S1S2 protein tagged with the donor and acceptor fluorophores, are similar and could be fit by a single exponential with time constants of $300 \pm 10 \mu\text{s}$ and $296 \pm 7 \mu\text{s}$, respectively, in the absence and presence of $5 \mu\text{M}$ RNA aptamer #25 (Figure 7A). The lack of an effect on the FRET lifetime suggests that the aptamer-bound form of the protein exhibits the same distance between the donor and acceptor as seen in the apo state. These results suggest that RNA aptamer #25 stabilizes the isolated ligand binding domain in an open cleft conformation similar to that observed for the apo state of the protein. To further confirm these observations, a competitive FRET experiment was performed in which the $5 \mu\text{M}$ glutamate was put in competition with $5 \mu\text{M}$ RNA aptamer #25, and the effect on the FRET lifetime was studied (Figure 7B). As previously reported, the FRET lifetime of the probe decreases in the presence of $5 \mu\text{M}$ glutamate relative to the apo state, consistent with a decrease in the donor:acceptor distance and cleft closure conformational change (Figure 7B) upon binding glutamate. The decrease in the FRET lifetime is reversed upon addition of $5 \mu\text{M}$ RNA aptamer #25 to the probe in the presence of $5 \mu\text{M}$ glutamate, with the lifetime being similar to that observed for the apo state. Thus, these data indicate that RNA aptamer #25 displaces the glutamate and changes the conformation from a closed cleft state to an open cleft state. These results clearly demonstrate that the structure of the RNA-bound protein should be similar to the open state structure observed for the small molecule antagonist-bound state of this receptor (Armstrong and Gouaux, 2000).

RNA reduces neuronal damage induced by OGD injury

Exposure of rat primary cortical neurons to RNA aptamer #25 or RNA pool zero (RNA pool 0) at concentrations of up to $0.1 \mu\text{M}$ or to CNQX at $30 \mu\text{M}$ for 24 h did not produce cytotoxicity, as determined with LDH and MTT assays (Figure 8A). While, pre-treatment of neurons with $0.1 \mu\text{M}$ but not with $0.02 \mu\text{M}$ of RNA aptamer #25, significantly reduced neurotoxicity associated with OGD injury as determined with LDH and MTT viability assays at 24 h after the onset of injury (Figure 8B). The extent of neuroprotection by $0.1 \mu\text{M}$ RNA aptamer was comparable to that of $30 \mu\text{M}$ CNQX, and this corresponds to the maximum protection that has been detected by CNQX towards OGD injury (supplemental Figure 3). In agreement with the LDH and MTT assays, the neuroprotective effect of RNA aptamer #25 was confirmed by cell counting of the damaged/dead neurons labeled by FDA-PI (Figure 9). RNA pool 0, used as negative control for aptamer #25, had no effect on neuronal survival, while $0.1 \mu\text{M}$ of RNA aptamer #25 showed similar protection against neurotoxicity as $30 \mu\text{M}$ of CNQX, an AMPA and kainate receptor antagonist (Figure 8B and 9).

Discussion

Here we have used the SELEX procedure to evolve water soluble RNA based antagonists towards the agonist binding site of the AMPA subtype of the glutamate receptors. Radioactive ligand binding studies and electrophysiological measurements show that the aptamers exhibit comparable nanomolar affinities and micromolar inhibitory properties similar to those of the currently known high affinity competitive antagonists (Table 3). It should be noted that typically higher affinities are observed in radioactive ligand binding studies relative to the electrophysiological investigations, and this observation is thought to be due to prolonged exposure of the receptor to the drug in the radioligand binding studies that could cause receptor desensitization, and perhaps internalization or damage to normal receptor function. The electrophysiological measurements on the other hand are rapid and able to probe the inhibitory effects on the open channel state of the receptor. While the activity for the RNA aptamer is

similar to the known antagonists, these RNA-based ligands have an advantage that they are water-soluble. The RT-PCR products typically yield 10 µl of 0.5 mM and at this concentration no visible precipitate was observed.

To study the structural consequence of RNA aptamer binding to the ligand binding domain of the glutamate receptor we have used fluorescence resonance energy transfer measurements. These data show that the RNA aptamer stabilizes the AMPA receptor ligand binding domain in an open state similar to that observed in the structures of quinoxaline bound forms. This observation coupled to the fact that three of the RNA aptamers with the consensus sequence but differing sequences on either side of the consensus sequence bind with similar affinity to the GluR2 receptors suggests that the bases in the consensus sequence are involved in binding and in keeping the ligand binding domain in an open state, and thus inhibiting the receptor. Future experiments with smaller constructs would allow us to determine the minimum construct required for high affinity binding.

In addition to characterizing the structural and functional consequences of the RNA aptamer binding to the AMPA receptors, here we also show preliminary evidence that the RNA aptamers significantly reduce neurotoxicity associated with OGD injury in embryonic rat cortical neurons. The embryonic rat cortical neurons express the Ca^{2+} permeable AMPA receptors and are hence excellent models to study the blockade of excitotoxicity mediated by this class of receptors (Jonas and Burnashev, 1995). While the OGD studies demonstrate that the RNA aptamers exhibit neuroprotection against OGD, the extent of protection produced by 0.1 µM RNA aptamer #25 is comparable to that of 30 µM CNQX. The radioactive binding and electrophysiological studies (Table 3), on the other hand, show similar binding affinities and inhibitory properties for the two. One reason for the differences in the neuroprotective and inhibitory properties could be the fact that the RNA is selective for AMPA receptors, while CNQX can bind to both AMPA and kainate receptors which are expressed in cortical neurons (Dai et al., 2002). Additionally, it is possible that the RNA aptamers act on other mediators of excitotoxicity such as NMDA receptors, glutamate transporters, etc. Future experiments studying the neuroprotective properties of RNA aptamers in combination with specific antagonists for these proteins will allow for a more detailed correlation between the changes due to a specific receptor and neuroprotective properties.

While, we cannot draw direct correlation between the electrophysiological measurements and the OGD neuroprotective properties, the present studies clearly establish that the RNA aptamers evolved are competitive antagonists of the AMPA receptors and show significant neuroprotection against OGD mediated injury. These properties of the RNA aptamers coupled to the fact that these are water-soluble ligands make them promising excellent candidates for potential use as neuroprotective drugs. While, it is still unknown as to whether these RNA aptamers can cross the blood brain barrier, this may not be an issue since newer vehicles are being developed that could deliver drugs such as RNA across the blood brain barrier.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The binding affinity of RNA SELEX pool 8 to GluR2-S1S2 using glutamate as a competitor. The IC_{50} for the displacement of bound RNA by glutamate obtained by fitting the data to the Equation 1 was $1.0 \pm 0.2 \mu M$.

Figure 2.

The functional effect of RNA aptamers on AMPA receptors and kainate receptors. The whole cell currents were evoked by (A) 5 μ M aptamer #25, (B-F). 250 μ M glutamate in the absence (solid line) and presence of 1 μ M aptamer #25 (dashed line), (G). 250 μ M glutamate in the absence (solid line) and presence of 1 μ M aptamer #10 (dashed line).

Figure 3. Inhibition of AMPA receptors by RNA aptamers with the consensus motif. Bar graph showing fraction of whole cell current mediated by 250 μ M glutamate inhibited by 1 μ M aptamer in AMPA and kainate receptors.

Figure 4.

The dose response curve for GluR2-flip receptors activation in the absence and presence of the RNA aptamer. The whole-cell currents from GluR2-flip receptors were evoked by increasing concentrations of glutamate in the absence (square) and presence of various concentration of aptamer # 25, 500nM (open square), 1 μM (circle) and 2.5 μM (open circle).

Figure 5.

Schild plot showing the inhibition of GluR2-flip receptor by RNA aptamer #25. The changes in the EC_{50} values for the three concentrations of RNA show a linear dependence with a slope of -0.99 and x-intercept of 6.11 ± 0.06 .

Figure 6.

Dose dependence of RNA aptamer inhibitory activity. The whole cells currents from GluR2-flip receptors were evoked by 250 μ M glutamate in the presence of various concentration of aptamer #25. The approximate IC_{50} for the aptamer is $1.0 \pm 0.1 \mu$ M.

Figure 7.

The structural consequences of RNA aptamer binding. The sensitized-emission FRET lifetimes for GluR2-S1S2-T394C-S652C labeled with fluorescein and terbium chelate were measured (A) in the absence (blue) and presence of 5 μ M aptamer #25 (pink); (B) in the apo state (green), in the presence of 5 μ M glutamate (blue), and in the presence of 5 μ M glutamate plus 5 μ M aptamer #25 (red).

Figure 8.

Neuronal injury and viability measured by LDH and MTT Assays. Twelve-day old primary cortical neurons were incubated with RNA aptamer #25 or the initial RNA pool (RNA pool 0) at 0.02 and 0.1 μ M, or CNQX (30 μ M) starting 30 min prior to OGD and during the subsequent 24 h of re-oxygenation. (A) Absence of toxicity of these compounds at the investigated concentrations was determined in the cultured neurons without OGD. (B) Neuroprotective effect of aptamer #25 was determined at 24h after OGD injury. The increased LDH release and reduced MTT value signify the loss of neuronal integrity. The data were expressed as mean $\pm$ SEM (n=6). * $P \leq 0.05$ compared with the control, ^ $P \leq 0.05$, compared with OGD groups.

Figure 9.

Neuronal survival/death was measured by FDA-PI. Representative photographs of FDA-PI labeled neurons are presented in (a-e) and bar graph displaying the percentage of damaged/dead neurons at 24h after OGD. Nuclear uptake of propidium iodide (PI; red) and somal loss of fluorescein diacetate (FDA; green) indicate the loss of cell integrity. Vehicle (b), 0.1 μ M aptamer #25 (c), 0.1 μ M RNA pool 0 (d) or 30 μ M CNQX (e) were added into the culture media 30 min prior to OGD and incubated for 24 h. The data are presented as mean $\pm$ SEM (n=4). * $P \leq 0.05$, compared with all other groups.

Table 1
Consensus motif of glutamate displaceable RNA aptamers (5'-3')

Aptamer #	Sequence
1	ACCGCA <u>CCCCCA</u> AUCUCUAUGAGAUUUUACAGUGCGGU
3	AUUCUACUAACCU <u>CCCCCA</u> GUACUAUGGGACAUAACCA
5	UUAUCUUCUCAUGUUAGUACGU <u>ACCUC</u> UAAGGACUACG
9	ACAUAUUUGUCAA <u>CACUCCA</u> UCCCCGUCGUACUCACUU
10	AUUUCGUAAAGAUUCGCCUGUACUACA <u>UCCCCA</u> AU
11	UUAUCACCUAAGGACCAUCCUUCUACCCAUGUG <u>CCCCACA</u> C
13	AACAUCUCUGAUACAAAUUUAUUGUCC <u>UUUCCCA</u> C
22	AACAUCUUAUU <u>UCCACA</u> CCCCGUACACUGUCCACAAC
24	UACGCAACACCUCUGCCAACCGUAGUAUAUUA <u>CCCCCA</u>
25	CGGCGAACUACCUAA <u>CCCCCA</u> CAAUUGUAUUUACGCC
30	ACCU <u>UCCCCA</u> ACUAGUUCUUGCGUACUACUUACGCCAC
32	CAUCAUUAUC <u>UCCUCA</u> UAGUGCUUCGGUUAACAUCUC
37	UCCGCA <u>CCCCCA</u> AUCUCUAUGAGAUUUUACAGUGCGGU
40	UCCAAACCCACU <u>CCCCCA</u> ACACUACCUUCCCGCAAACAUC

Table 2A

The binding affinities of RNA aptamers for GluR2-flip receptors

RNA aptamers	GluR2-flip
#1	29 ± 7
#10	42 ± 16
#25	37 ± 5

Table 2B

The binding affinities of RNA aptamer # 25 for AMPA and GluR6 receptor

Receptor	RNA aptamer # 25
GluR1-flip	189 ± 13
GluR3-flip	47 ± 4
GluR4-flip	71 ± 15
GluR6	>200

Table 3

Comparison of the binding affinities (K_d) as determined from radioactive binding, and inhibitory properties ($pA2$) as determined from the intercept of the Schild plot for AMPA receptor antagonists

ligands	K_d (nM) ^b	$pA2$ ^c
Aptamer #25 ^a	37 ± 5	6.11 ± 0.06
YM872 ^b	96 ± 2.4	6.97 ± 0.01
YM90K ^b	71 ± 2.0	6.83 ± 0.01
NBQX ^b	46 ± 0.79	7.24 ± 0.01
CNQX ^{b and c}	250 ± 12	6.39^c

^a for GluR2-flip receptors

^b from ref (Kohara et al., 1998) for AMPA receptors expressed in oocytes. The RNA for AMPA receptor expression is from rat cerebral cortex.

^c from ref (Paternain et al., 1996)