RNA Aptamers Selected against the GluR2 Glutamate Receptor Channel†

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ABSTRACT: The excessive activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, a subtype of glutamate ion channels, has been implicated in various neurological diseases such as cerebral ischemeia and amyotrophic lateral sclerosis. Inhibitors of AMPA receptors are drug candidates for potential treatment of these diseases. Using the systematic evolution of ligands by exponential enrichment (SELEX), we have selected a group of RNA aptamers against the recombinant GluR2Q_{flip} AMPA receptor transiently expressed in HEK-293 (human embryonic kidney) cells. One of the aptamers, AN58, is shown to competitively inhibit the receptor. The nanomolar affinity of AN58 rivals that of NBQX (6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione), one of the best competitive inhibitors. Like NBQX, AN58 has the highest affinity for GluR2, the selection target, among all AMPA receptor subunits. However, AN58 has a higher selectivity for the GluR4 AMPA receptor subunit and remains potent even at pH = 6.8 (i.e., a clinically relevant acidic pH), as compared with NBQX. Furthermore, this RNA molecule possesses stable physical properties. Therefore, AN58 serves as a unique lead compound for developing water-soluble inhibitors with a nanomolar affinity for GluR2 AMPA receptors.

α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)[1] receptors of the glutamate ion channel family (*1*) are an important target of drug development, like the *N*-methyl-D-aspartate (NMDA) receptor of the same family, for the treatment of neurodegenerative diseases (*2*). At the receptor level, an AMPA receptor opens its channel in response to the binding of glutamate on the microsecond (*μ*s) time scale, much faster than NMDA receptor channels (*3*−*6*). The opening of the AMPA channel is thought to provide the initial membrane depolarization, thus enabling the NMDA channel to function by relieving the magnesium block (*3*, *7*). AMPA receptors have four subunits: GluR1−4. The assembly of the same or different subunits, presumably in tetramers (*8*), produces functional ion channels (*9*, *10*). The RNA editing at the Q/R (i.e., glutamine/arginine) site of GluR2 is an important mechanism of controlling calcium permeability of the receptor in that AMPA receptors with the unedited GluR2 (the Q form) are substantially calcium permeable, whereas those with the edited GluR2 (the R form) are not (*10*−*14*). Extra calcium entry into neurons through excessively activated AMPA receptors causes intracellular calcium overload, which in turn initiates cell death signaling pathways (*15*). Therefore, developing AMPA receptor inhibitors to control excessive receptor activity has been a long-pursued therapeutic strategy.

Traditionally, synthetic chemistry has been the main approach in making small-molecule inhibitors. This strategy has yielded a large number of various types of AMPA receptor inhibitors, such as quinoxalines, dihydrophthalazine derivatives, and 2,3-benzodiazepine compounds. Although AMPA receptor inhibitors with nanomolar affinities have been synthesized, poor water solubility has been a serious problem that so often plagues the clinical usefulness of these compounds. 6-Nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX), a classic competitive inhibitor of AMPA/kainate receptors (*16*), is an example of such a compound (*17*). To improve water solubility, a number of newer competitive antagonists were later synthesized (*18*−*20*). However, for 2,3-benzodiazepine derivatives, a class of noncompetitive AMPA receptor inhibitors developed with the most intense interest and effort to date (*2*), water solubility remains a major problem. Furthermore, to make new inhibitors by organic synthesis, slight chemical modifications of a previous template are often designed. The presumed effect of each modification must be tested before newer modifications can be introduced. Thus, the use of synthetic chemistry to prepare new inhibitors entails a template-based, stepwise process.

In the present study, we take a different approach in developing AMPA receptor inhibitors. The inhibitors we report here are aptamers, which are RNA molecules. Aptamers are selected from an RNA library using an in vitro iterative selection procedure known as systematic evolution of ligands by exponential enrichment (SELEX) (*21*, *22*). RNA aptamers are water soluble by nature and can fold into specific tertiary structures that confer high affinity and specificity against biological targets that even do not exist

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[1] Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; HEK-293 cells, human embryonic kidney cells; SELEX, systematic evolution of ligands by exponential enrichment; TAg, large T-antigen.

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in nature (*23*, *24*). Unlike conventional, template-based drug design, SELEX can produce lead compounds without templates. The lead compounds can be used to design newer, biostable RNA aptamers for both highly compartmentalized intracellular expression (*25*) and extracellular applications (*23*, *26*, *27*), such as the inhibition of AMPA receptors. Additionally, aptamers can be used as novel structural templates for the chemical synthesis of new inhibitors/drugs.

EXPERIMENTAL PROCEDURES

Receptor Preparation. Each of the AMPA receptor subunits, GluR1−4 (all flip variants), and the GluR6Q kainate receptor subunit was transiently expressed in the human embryonic kidney cells (HEK-293S) using a calcium phosphate protocol (*3*). The SV40 large T-antigen (TAg) gene was cotransfected to enhance single-cell receptor expression (*28*). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a 37 °C, 5% CO_2, humidified incubator. Forty-eight hours after transfection, the cells were either harvested for SELEX or directly used for patch clamp recording. To test selected aptamers on hippocampal neurons containing endogenous glutamate receptors, 1 day old, postnatal Sprague−Dawly rats were dissected and the hippocampal neurons were cultured as described (*29*).

SELEX. The RNA library for SELEX was prepared as described (*30*). The library was dissolved in the extracellular buffer (at a final concentration of 20 μM) containing (in mM) 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4). The fragmented HEK-293 cell membrane containing the $GluR2Q_{flip}$ receptor was prepared by homogenizing the cells using a 50 mM Tris acetate buffer (pH = 7.4) containing 10 mM EDTA and 1 mM phenylmethanesulphonyl fluoride, followed by centrifugation to collect the membrane fragments. The membrane-bound receptor was adjusted to a final concentration of 50 nM as determined by [^{3}H]AMPA binding. The reaction mixture was incubated at 22 °C for 40 min for RNA binding in the presence of 0.3 units/μL RNase inhibitor (Ambion). The desired RNAs were eluted using 1 mM NBQX and were then subject to reverse transcription and PCR.

Binding Assay. The binding affinity of putative RNA aptamers was measured by competition binding to the S1S2 soluble extracellular binding portion of the GluR2 receptor (*31*) in the presence and absence of NBQX. An RNA sample was internally labeled with [α-^{32}P]CTP (GE Health) and purified on a polyacrylamide gel electrophoresis (PAGE). The S1S2 protein was covalently linked to MagnaBind amine derivatized beads (Pierce). A binding reaction contained a mixture of 50 nM immobilized S1S2, 20 nM RNA, 6 ng/μL yeast tRNA (as a nonspecific competitor), and 10% DMSO in the extracellular buffer. [α-^{32}P]CTP-labeled RNA was mixed with ~40-fold excess amount of cold RNA sample. The background binding reaction contained additional 250 μM NBQX, since NBQX was used to evolve aptamers. All binding reactions were carried out at room temperature for 40 min. After binding, a sample mixture was loaded on to a dot-blot apparatus (Pierce) and washed using external buffer (3 × 200 μL/well). The radioactivity of the dot-blot binding was digitized in a phosphorimager (Typhoon Trio, GE Health), which was then quantified using ImageQuant TL (GE Health).

The K_d of an aptamer was estimated by nonlinear fitting of the binding data using eq 1

$$y = \frac{B_{max}\,x}{K_d + x} \quad (1)$$

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

RNA Purification. RNA aptamers were purified for quantitative assay. The purification involved an initial run through PAGE to obtain the wanted RNA aptamers generated from in vitro transcription. The aptamer sample was passed through a Q anion exchange column integrated with a Bio-Rad DuoFlow system to remove polyacrylamide. The buffer containing 25 mM Tris−HCl and 200 mM NaCl was run at a flow rate of 1.0 mL/min to remove polyacrylamide. The aptamer was eluted by passing a solution of 25 mM Tris−HCl and 1.5 M NaCl at the same flow rate, and then dialyzed in the extracellular buffer for assays. The removal of polyacrylamide in the RNA sample was confirmed by the 1D NMR spectrum both before and after passage through the Q column on a Bruker spectrometer operating at a ^{1}H frequency of 400 MHz. The proton resonance lines characteristic of acrylamide oligomers (~1.4−2.4 ppm and 7−8 ppm) were used to monitor polyacrylamide in the RNA sample (*32*).

Whole-Cell Recording. The procedures for whole-cell current recording was previously described (*3*). The cells used for recording were prepared as previously described in the "Receptor Preparation" of the Experimental Procedures. Briefly, an Axopatch-200B amplifier (Axon Instrument) was used in whole-cell recording at a cutoff frequency of 2−20 kHz by a built-in, 8-pole Bessel filter and digitized at a 5−50 kHz sampling frequency by a Digidata 1322A (Axon Instruments). An electrode for whole-cell recording had a resistance of ~3 MΩ when filled with the electrode solution containing (in mM) 110 CsF, 30 CsCl, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). All reagents were dissolved in the extracellular buffer. When aptamers were included, the buffer further contained 0.05 units/μL RNase inhibitor (final concentration). A flow device (*33*) was used to apply glutamate in the absence and presence of aptamers to a cell expressing the receptor of interest. The same recording protocol was used for kainate with GluR6 and for NMDA with hippocampal neurons (*29*). Unless noted otherwise, each data point represented an average of at least three measurements collected from at least three cells. All whole-cell recordings were at −60 mV and 22 °C.

Origin 7 was used for data analysis and plotting. Uncertainties reported refer to standard deviation from the mean unless otherwise noted. Student's t tests were preformed, as indicated in the corresponding data sets and figures. A P value less than or equal to 0.05 was considered significant.

RESULTS

Transiently Expressed $GluR2Q_{flip}$ AMPA Receptor as the SELEX Target. For the target of aptamer selection, the GluR2 subunit we chose was the unedited, alternatively spliced "flip" isoform or $GluR2Q_{flip}$. The choice of this subunit and the specific receptor isoform was based on the finding that

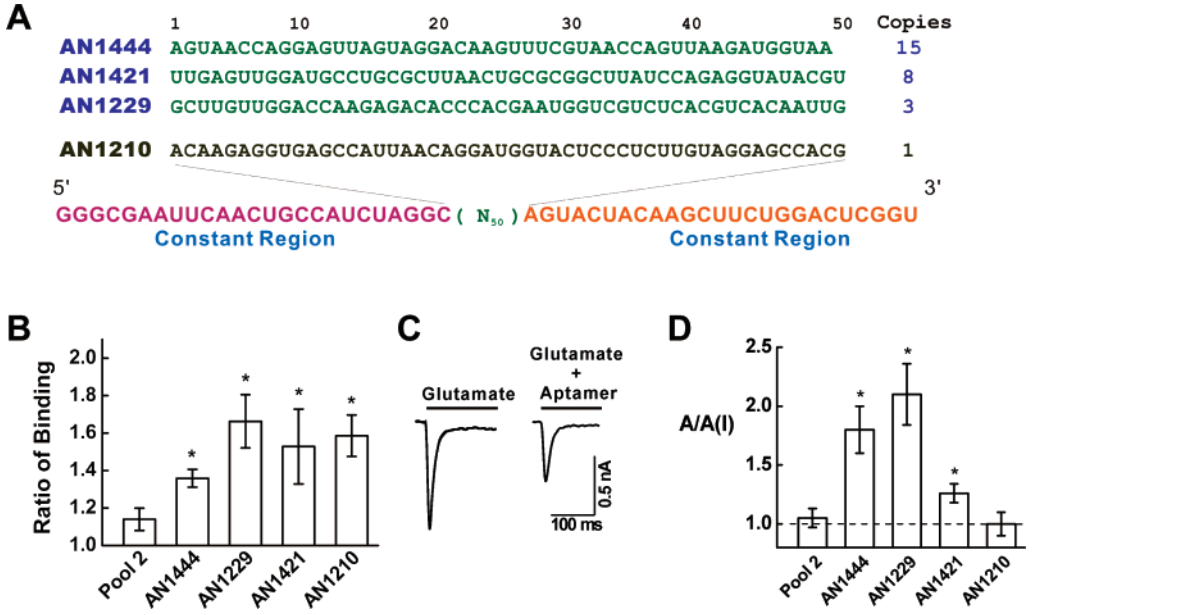

FIGURE 1: Three selected aptamers inhibit GluR2Q$_{flip}$ AMPA receptors. (A) The three RNA sequences of the variable region (N50) were isolated from a total of 80 clones from rounds 12 and 14; overall, 14 rounds were carried out, including three negative selections (i.e., rounds 5, 10, and 13). The arbitrary standard of selecting a "hit" for binding and functional assays was that each sequence at least repeated its appearance three times among 80 clones. The name for each sequence is on the left, whereas on the right is the number of isolated individual sequences. The sequence of the constant region is displayed at the bottom. AN1210 whose sequence is listed as well is thought to bind to, but does not inhibit, the receptor. (B) The radioactivity was measured from the [α-^{32}P]CTP-labeled RNA binding to the S1S2 partial receptor (see text) in the absence and presence of NBQX, the selection pressure, and the ratio of the radioactivity was plotted (∗ indicates $P \leq 0.05$ from the two-tailed Student's t test; H_0: $\mu = \mu_0 = 1$, 1 being the theoretical value of no binding here or no inhibition as in (D) below). For pool 2, $P = 0.0553$. (C) The whole-cell current response of GluR2Q$_{flip}$ to 500 μM glutamate was reduced in the presence of 150 nM AN1444. (D) The three aptamers selected inhibited GluR2Q$_{flip}$, shown as the ratio of the whole-cell current response in the absence and presence of 100 nM aptamer or $A/A(I)$. Unless otherwise noted, 500 μM glutamate was used here and in all measurements in this study. The inhibition of these aptamers was verified by using a 300 nM control, i.e., either pool 2, the second-round library, or AN1210, a sequence different from any of the three sequences. For AN1210, no detectable inhibition was observed even at concentrations up to 700 nM.

GluR2Q$_{flip}$ expression is aberrant in some neurological diseases. For example, in patients with amyotrophic lateral sclerosis (ALS), Q/R editing in the motor neurons is only 56% complete as compared to nearly 100% in the control (*34*). This significant, neuron-specific RNA editing defect is thought to be linked to selective motor neuron degeneration, a pathogenic hallmark in ALS (*34*). Similarly, the GluR2 Q/R editing defect is also found in pathological tissues of other neurodegenerative diseases, such as the prefrontal cortex of those with Alzheimer's disease and the striatum of those with Huntington's chorea (*35*). Furthermore, unlike the edited R form, the unedited Q form readily tetramerizes into functional AMPA receptors traffic to synapses (*36*). The expression of the alternatively spliced flip variant of GluR2, as compared to the flop, is also increased in the spinal motor neurons of ALS patients (*37*). The flip isoform of GluR2 is known to desensitize at least 3 times more slowly than the flop (*38*). Consequently, cells that contain more slowly desensitizing flip channels, such as motor neurons, are thought to be more vulnerable to excitotoxicity. These findings suggest that GluR2Q$_{flip}$ is a key AMPA receptor subunit/isoform in mediating calcium-induced excitotoxicity.

For the target preparation, we expressed GluR2Q$_{flip}$ transiently in HEK-293S cells, using a standard calcium phosphate protocol. To maximize the cell-surface receptor density by the transient transfection method, we coexpressed SV40 TAg with GluR2Q$_{flip}$ in the same cell (*28*). Furthermore, we prepared the membrane fragments harboring intact GluR2Q$_{flip}$ receptors for SELEX. In the successful selection described below, the receptor density was ~0.61 pmol/mg of cell mass (i.e., fragmented membrane). To minimize the selection of unwanted "aptamers" toward other targets such as lipids and other proteins in the cell membrane, negative selection was included such that the HEK-293S cell membrane harboring no GluR2Q$_{flip}$ (but with TAg) was used to filter off unwanted RNAs from enriched RNA libraries (see the legend of Figure 1).

Selection of Aptamers. A total of 14 rounds of SELEX cycles, including 3 rounds of negative selection, were carried out against GluR2Q$_{flip}$ from a regular or unmodified RNA library containing ~10^{15} RNAs (*30*). After cloning and sequencing, we identified three dominant sequences (Figure 1A). To determine whether these sequences corresponded to inhibitory RNA molecules, we first ran a radioactivity binding assay using the ^{32}P-labled RNAs (see Experimental Procedures), including two additional controls, the RNA library from cycle 2 (pool 2) and AN1210 RNA, a single sequence identified from cycle 12 but without any sequence homology with the rest of the clones (Figure 1A). It appeared that even pool 2 showed some binding affinity (Figure 1B), albeit the lowest among the tested sequences. The presumed affinity exhibited in this radioligand-binding experiment could have originated from specific and/or nonspecific binding to the receptor. Specific binding refers to an aptamer bound to the agonist binding site because NBQX, a classic competitive inhibitor (see further description below), was used to evolve these aptamers. In addition, we used the S1S2 protein, an extracellular portion of GluR2, in the binding

assay, instead of the holo-receptor embedded in lipid membrane, for the purpose of reducing the nonspecific binding of RNA to lipid membrane.

To specifically characterize the biological activity of the selected RNA, we carried out whole-cell recording to directly assay whether an RNA was capable of inhibiting GluR2Q_{flip}. We reasoned that if an RNA was an inhibitor, as determined by the radioactivity assay (Figure 1B), it would be expected to inhibit the whole-cell current through the GluR2 channel, expressed in HEK-293S cells. Indeed, we found that all three sequences inhibited the activity of the GluR2Q_{flip} channel as evidenced by the reduction of the whole-cell current response to glutamate (Figure 1, parts C and D). In contrast, neither pool 2 nor AN1210 showed any inhibition (Figure 1D). In light of both binding (Figure 1B) and whole-cell recording data (Figure 1D), we also concluded that AN1210 (Figure 1A) could bind to the receptor but the binding did not cause inhibition.

Sequence Characterization. To begin to understand the structure−function relationship of these RNA aptamers, we first compared the three RNA sequences (Figure 1A). However, we found no apparent sequence similarity in the form of short-stretched "consensus sequences", commonly observed as a SELEX outcome. A lack of short-stretched consensus sequences suggested that an aptamer may fold into a larger structural entity to act as an inhibitor. Other possibilities certainly exist, such as that an insufficient number of sequences were sampled for this comparison. In an attempt to address these possibilities, we focused on one individual aptamer, AN1444 (AN1444 was both more relatively abundant and potent) (Figure 1, parts A and D). We first explored whether the full sequence of AN1444 could be reduced into a shorter but fully functional sequence. Guided by the secondary structure prediction using the Mfold program (*39*), the full sequence of AN1444 was reduced to a 58 nucleotide (nt) long sequence with full activity (Figure 2A). One of the secondary structures predicted from the Mfold program is shown (Figure 2B). Removal of 26 nt from the 5′ direction (a constant region of the sequence template) from the original length resulted in a total loss of inhibitory function, suggesting that this segment played an essential role for the aptamer function. In contrast, progressive truncation of the sequence from the 3′ end showed that the 58 nt version or AN58 retained activity, whereas shorter versions, such as 53 nt, 49 nt, or 46 nt, did not (Figure 2A). The deletion of five or more nucleotides at the 3′ end of the 58 nt version presumably disrupted critical base pairing (Figure 2B) as predicted by Mfold (*39*). Interestingly, a lack of short-stretched consensus sequences was also reported in aptamers selected against human epidermal growth factor receptor 3 (*40*) and the TATA-binding protein (*30*). Thus, AN58 became our working template for the subsequent functional studies described below and the ongoing structural study.

Functional Characterization of Aptamer AN58. We have characterized the biological properties and activities of AN58 in ways described below (Figure 3) and compared them with NBQX. The comparison with NBQX is based on the fact that NBQX was used as the selection pressure to evolve the aptamers and NBQX is one of the most potent competitive inhibitors discovered to date (*16*). First, we performed the radioligand displacement binding experiment with AN58

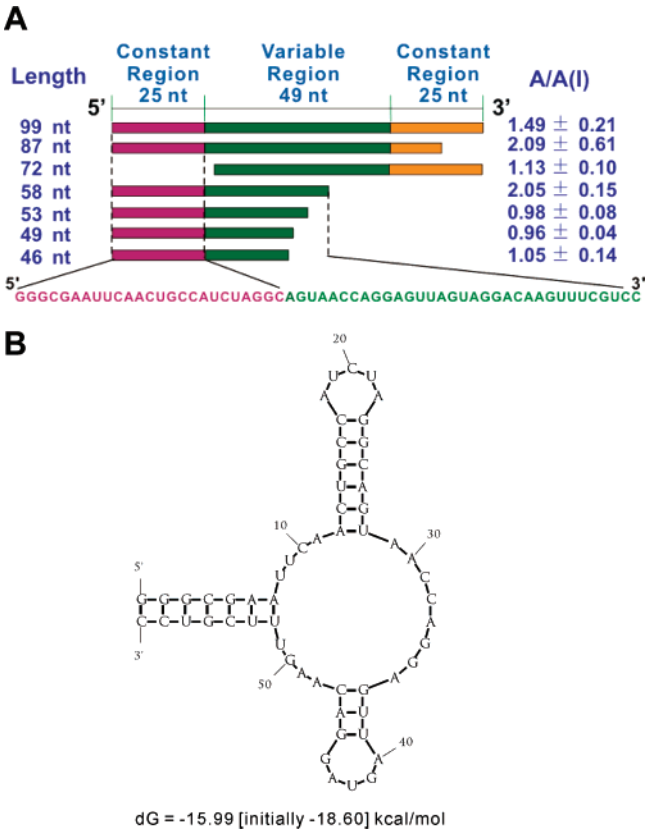

FIGURE 2: AN1444 was reduced to a 58 nt long, functional aptamer (AN58). (A) The original 99 nt AN1444 was truncated from both 5′ and 3′ directions. The corresponding shorter versions were tested using whole-cell recording with HEK-293S cells expressing the GluR2Q_{flip} receptor, and the results are reported as *A*/*A*(*I*). (B) A representative secondary structure of AN58 predicted by Mfold.

using the S1S2 ligand-binding core of GluR2 in the presence and absence of saturation of NBQX (Figure 3A). A K_d value of 0.419 ± 0.221 nM was estimated using eq 1 (Experimental Procedures). This value is more than 47-fold higher than the K_d of NBQX binding to the GluR2 S1S2 previously reported (*41*), suggesting that AN58 is a highly potent inhibitor. Second, we also measured the dose−response relationship of glutamate with the GluR2Q_{flip} channel expressed in HEK-293S cells in the absence and presence of AN58 (Figure 3B). The fact that the dose−response curve was right-shifted in the presence of AN58 and eventually converged at saturating concentrations of glutamate was also supportive of AN58 being a competitive inhibitor. This is plausible because NBQX-displaced RNA aptamers were supposedly bound to the agonist binding site or to a mutually exclusive site(s) on the receptor. In fact, the dose−response curve of AN58 was similar to that of NBQX (data not shown). Using whole-cell recording, we further determined that AN58 inhibited the GluR2Q_{flip} channel in a concentration-dependent manner, yielding an IC_{50} value of 121 ± 7 nM (Figure 3C).

Next we determined the specificity of AN58 for all AMPA receptor subunits and a representative kainate subunit (i.e., GluR6) by testing its inhibition on each homomeric channel expressed in HEK-293S cells (Figure 3D). AN58 inhibited all AMPA receptor subunits. The specificity of AN58 for these receptors was found to be comparable with NBQX, except for GluR4, where AN58 showed a higher affinity for reasons not yet known. In all AMPA receptor subtypes tested,

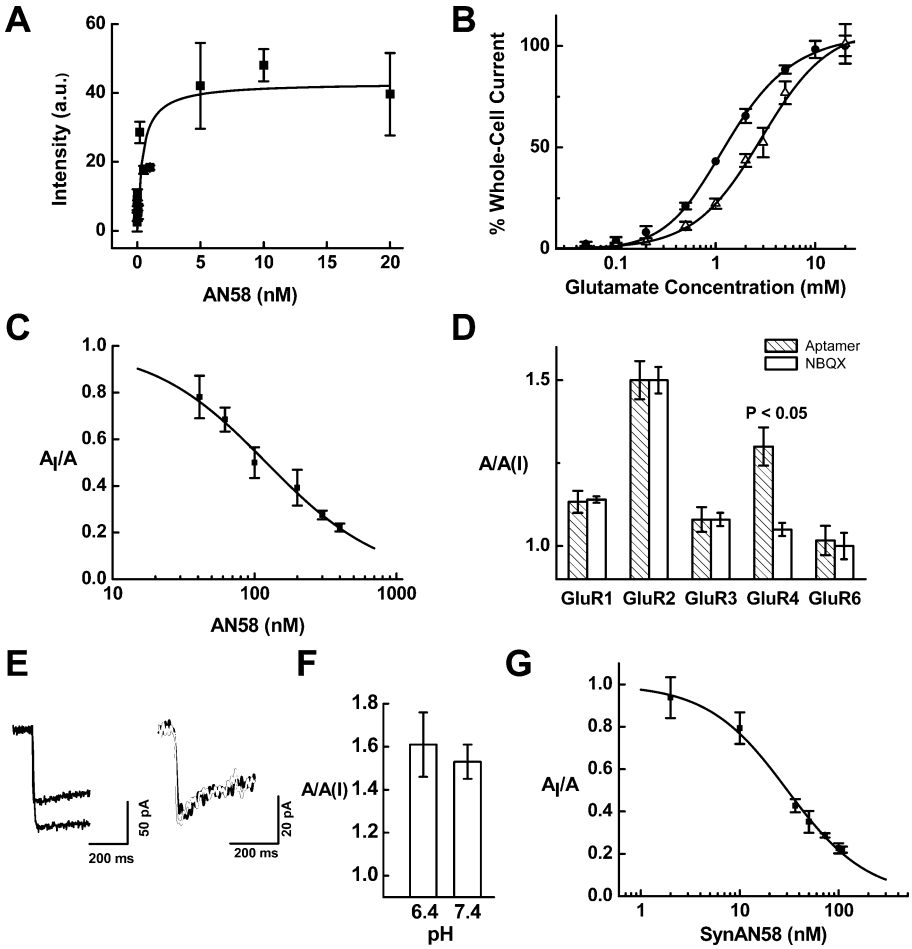

FIGURE 3: Comparison of inhibitory properties of aptamer AN58 with NBQX. (A) Radioligand displacement assay of AN58 with S1S2. Each point represents the average intensity in arbitrary units of three radioactivity measurements of specific binding. The K_d was determined to be 0.419 ± 0.221 nM by nonlinear fitting of the binding data using eq 1. (B) AN58 inhibited the $GluR2Q_{flip}$ by a parallel shift of the dose−response curve in the presence of 150 nM AN58, consistent with a competitive mechanism. (C) The ratio of the whole-cell current recording in the presence and absence of AN58 with HEK-293S cells expressing the $GluR2Q_{flip}$ channels. An IC_{50} value of 121 ± 7 nM was obtained by fitting the current amplitude as a function of the AN58 concentration to the Hill equation (*55*). (D) Specificity of AN58 to AMPA and GluR6 kainate receptors. Here, the specificity of both NBQX and AN58 was normalized against the GluR2Q response. To ensure the inhibition was comparable, the glutamate concentration was chosen to be equivalent to ∼25% of the faction of the open channel or roughly about half of the EC_{50} value for a particular channel. Specifically, the glutamate concentration was 100 μM for GluR1 and GluR6 and 500 μM for GluR2−4, whereas the aptamer concentration was kept at 150 nM (*3*, *4*). AN58 showed higher affinity to GluR4 ($P \leq 0.05$ from a two-sample Student's t test, as compared with NBQX; H_0: $\mu_1 = \mu_2$). (E) AN58 inhibited endogenous AMPA receptors (left panel) in rat hippocampal neurons, but was ineffective on NMDA receptors (right panel). The left panel shows the whole-cell current response, from the same neuron, induced by 300 μM kainate in the absence (the lower trace) and presence of 150 nM AN58 (the upper trace). The right panel shows the whole-cell current response, induced by 1 mM NMDA and 100 μM glycine in the absence (black) and presence of 150 nM AN58 (gray). (F) AN58 remained potent at pH = 6.4. All other conditions used for this experiment were the same as in (D) except that the $GluR2Q_{flip}$ receptor was used exclusively for this assay and the extracellular buffer was adjusted to be the corresponding pH. The same buffer was used to dissolve AN58 for this assay. (G) The IC_{50} value for synthetically prepared AN58 or SynAN58 was determined to be 30 ± 1 nM from the fit of the whole-cell current amplitude to the Hill equation (*55*) as a function of the SynAN58 concentration.

AN58 exhibited the highest specificity to GluR2 subunit, presumably because GluR2 was the target of selection. However, neither AN58 nor NBQX inhibited the GluR6 kainate receptor at the concentration tested. This is desirable because kainate and AMPA receptors have different functions (*42*). Furthermore, AN58 was tested with hippocampal neurons, because these cells contained various endogenous glutamate receptors and are considered a classical paradigm for testing excitatory neurotransmission (*43*). With the use of kainate as the nondesensitizing AMPA receptor agonist (*43*) to evoke the whole-cell response, AN58 inhibited endogenous AMPA receptors, as expected (left panel in Figure 3E). In contrast, AN58 was ineffective in inhibiting NMDA receptor response (right panel in Figure 3E), consistent with this "inherited property" from NBQX, a non-NMDA receptor inhibitor, and the fact that the full length predecessor of AN58 and all other discovered aptamers (Figure 1A) were not evolved against NMDA receptors.

AN58 further retained the inhibitory potency when the pH dropped from 7.4 to 6.4 (Figure 3F). Under the same pH condition, NBQX was reported to lose its potency by >3-fold (*17*). This acidic pH is clinically linked to the infarcted brain regions (*17*), thus making the efficacy of inhibition by a potential drug at this acidic pH a critical requirement for effective stroke treatment. The full activity of AN58 may be attributed to the excellent stability of RNA under this slightly acidic condition (*44*). Furthermore, AN58 is stable in that it remains fully active even after ethanol precipitation,

heating for >10 min at 70 °C, and/or storage in the frozen state for more than a year.

Although the IC_{50} value of AN58 is in the nanomolar range, which is clearly desirable as compared with a large number of inhibitors prepared synthetically (*2*), the sequence identified from the RNA library may not confer the most optimized structure. To find a more optimal structure with a higher affinity, we tested chemically synthesized AN58. We reasoned that the synthetic AN58 might show a different conformation(s) and/or structure(s), because the synthetic RNA was made from the 3′ to 5′ direction, different from that of the enzymatic transcription. Indeed, by the whole-cell recording assay, the synthetic version of AN58 was found to have an IC_{50} of ∼30 nM (Figure 3G). This potency rivals existing competitive antagonists for AMPA receptors, including NBQX, SPD 502 (*18*), ZK200775 (*19*), and YM872 (*20*). Furthermore, this result suggests the possibility that the post-SELEX selection using doped library and other modifications may yield additional aptamer sequences/structures with even higher potency. The use of a doped library or a partially randomized library constructed based on the initially selected sequences provides a systematic search of structures with stronger activities (*45*).

DISCUSSION

As an initial proof of concept, we have used SELEX against the recombinant $GluR2Q_{flip}$ receptor embedded in the membrane fragments of HEK-293S cells and successfully isolated a group of RNA aptamers. Our initial characterization of these aptamers focused on AN58. Its properties are compared favorably with those of NBQX. The nanomolar IC_{50} value of AN58 assayed using whole-cell recording with the holo-$GluR2Q_{flip}$ receptor in HEK-293S cells and the picomolar K_d value measured by radioligand displacement assay with the corresponding S1S2 ligand-binding core all suggest that AN58 rivals any existing AMPA receptor inhibitors currently known, including NBQX (*2*, *17*). AN58 shows broad activity for all of AMPA receptor subunits, similar to NBQX, but has no activity for either GluR6 or NMDA receptors. As predicted, AN58 has the highest affinity for the GluR2 subunit, the target of selection.

The broad activity of AN58 toward AMPA receptors is likely attributed to the nature of AN58 being a competitive antagonist for the glutamate site, and glutamate is the common agonist or the common neurotransmitter. Furthermore, AMPA receptor genes share ∼70% sequence homology (although the genes may undergo alternative splicing in both the C-terminus and the flip/flop region) (*46*). In the S1 extracellular binding domain, the sequence homology among AMPA receptors reaches more than 85%. However, AN58 does exhibit an improved affinity toward the GluR4 subunit, as compared to NBQX (Figure 3D). This result suggests that selection of aptamers that are more specific to an AMPA receptor subunit/conformation is possible, perhaps by using different types of compounds, such as noncompetitive inhibitors, as selection pressure.

One of the most important findings from this study is the demonstration of the possibility of developing an AMPA receptor inhibitor (i.e., AN58) that is equally potent, if not more potent than NBQX, and also water soluble. In addition, AN58 remains stable and potent even at a clinically relevant acidic pH, whereas NBQX loses its potency by 3-fold. These characteristics, coupled with its physical stability, suggest that AN58 is a possible alternative to its chemical counterpart NBQX.

The determination of the binding site of AN58 on the receptor and the structure of AN58 using biochemical and structural means will be useful in the future. This work shall not only provide additional insights into the presumed mechanism of action of AN58 but also complementary information about the receptor site and the structure of AN58 for future development of inhibitors. Furthermore, the aptamer itself can be used as a new template and/or reagent targeting GluR2 and even other AMPA receptors as a whole. For this purpose, the aptamer needs to be chemically modified first, such as changing 2′-OH to 2′-F, to become nuclease-resistant (*47*). In addition, other types of chemical modifications are available, which can be used for enhancing pharmacokinetics, immobilization, or labeling of aptamers to make them useful as reagents and potential drugs (*23*, *27*). For the latter, however, more work such as on the membrane permeability needs to be done.

The use of SELEX to evolve aptamers against a desired target generally requires target preparation. Various methods of target preparations have been developed (*48*), which have made SELEX increasingly routine, especially for soluble targets (*23*). However, preparation of a transmembrane protein target in both its entirety and functional form is still challenging. Consequently, the application of SELEX to aptamer selection against transmembrane proteins is limited as compared with SELEX with soluble targets. Membrane proteins often require a lipid environment to maintain wild-type functionality and may therefore have to be prepared and presented for SELEX in a complex background. As such, the evolution of aptamers can be dominated by an overwhelming amount of lipids and other targets in the lipid environment, as compared with the amount of target protein. In the successful SELEX cases involving membrane proteins, native membrane tissues (i.e., *Torpedo californica* electroplax membrane and rat forebrain membrane) have been used for RNA aptamer selection (*49*, *50*). Live cells have been used for selection of ssDNA aptamers, including human red blood cell membrane (*51*) and hematopoietic tumor cells (*52*). In all the cases described above, proteins used for SELEX are native to cell membrane and these proteins are likely rich with respect to lipid quantity such that the SELEX are successful in these target-enriched environments. For recombinant membrane proteins not found in native tissues, a cell line (*53*) or a partial, soluble portion of a membrane protein (*40*, *54*) has been constructed for SELEX.

Here we demonstrate that $GluR2Q_{flip}$, which may not be found in a pure isoform in a native tissue, can be transiently expressed in HEK-293S cells using a conventional transfection protocol and that the membrane fragments harboring the holo-receptor can be readily used as a SELEX target for the evolution of aptamers from a complex membrane background. It should be pointed out that the success of SELEX in our case correlated to the receptor density of ∼0.61 pmol/mg of cell mass (i.e., in HEK-293S cells), which was achieved by coexpression of TAg (*28*). We did not, however, run the SELEX without TAg and therefore did not know whether a receptor density lower than what we used would have worked or not. Nevertheless, the use of HEK-

293S cells for transient expression of an ion channel, and other transmembrane proteins, is already a widely popular and routine method of receptor protein preparation for various assays. In comparison with the use of cell lines permanently expressing the protein of interest, transient expression is simpler and time saving. The use of membrane fragments harboring the protein of interest instead of live cells avoids the necessity of using chemically modified, nuclease-resistant RNA libraries. Therefore, our method entails minimal procedures to add to normal protocols for both membrane protein expression in cells and target preparation for SELEX.

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