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Potent and Selective Inhibition of the Open-Channel Conformation of AMPA Receptors by an RNA Aptamer†

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Abstract

Inhibitors of AMPA receptors are useful as biochemical probes for structure-function studies and as drug candidates for a number of neurological disorders and diseases. Here we report the identification of an RNA inhibitor or aptamer by an in vitro evolution approach. Using a laser-pulse photolysis technique, we further characterized the mechanism of inhibition of this aptamer on the AMPA receptor channel-opening rate process in the microsecond-to-millisecond time domain. Our results show that the aptamer we isolated is a noncompetitive inhibitor that selectively inhibits the open-channel conformation of AMPA receptors with nanomolar affinity. The potency and the selectivity of this noncompetitive aptamer rival those of small molecule inhibitors. Our results therefore demonstrate the utility of this approach to develop water-soluble, highly potent and conformation-selective noncompetitive inhibitors of AMPA receptors.

A protein is generally dynamic and adapts a specific conformation for function (1-2). Using molecular agents that bind selectively to a specific protein conformation among its conformational repertoire is thus a powerful means to exert a tighter molecular recognition to more effectively regulate the function of that protein, and to even engineer a new protein function. For instance, small chemical compounds have been found to stabilize a conformation for some apoptotic procaspases to induce autoproteolytic activation of these proenzymes (3). Catalytic antibodies have been created, based on transition-state structural analogs, to accelerate chemical reactions by stabilizing their rate-determining transition states along reaction pathways (4).

Here we describe the discovery of RNA inhibitors or aptamers that selectively target the open-channel conformation of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)1-subtype glutamate ion channel receptors. The open-channel conformation exists in the time span of microsecond to a few milliseconds (5), after the receptors bind to glutamate, the endogenous neurotransmitter; even in the millisecond time scale, the glutamate-bound receptors turn into the desensitized, channel-closed receptor form (6). Only through the transient, open-channel conformation of the postsynaptic receptors is a nerve impulse transmitted in the central nervous system. AMPA receptors are indispensable for

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SUPPORTING INFORMATION AVAILABLE

The supporting information includes a schematic diagram showing SELEX, additional data showing that AG1407 in full length inhibited GluA2Q_{flp} receptor more strongly as glutamate concentration increased, and additional data showing that although weak, AG56 inhibited the open-channel conformation of GluA4 receptor subunit. Furthermore, we also provide an additional trace to show AG56 did not additionally inhibit the k_{obs} more than it did to the k_{cl} ; yet this trace had a higher percentage of whole-cell current reduction. This material is available free of charge via the Internet at <http://pubs.acs.org>.

brain development and activity such as memory and learning (7-10), whereas excessive activation of AMPA receptors, which leads to an intracellular calcium overload, has been implicated in various neurological diseases, such as cerebral ischemia and amyotrophic lateral sclerosis (11). Developing inhibitors to control excessive receptor activity has been a long pursued therapeutic strategy for a potential treatment of these neurological disorders and diseases.

To find inhibitors selectively targeting the open-channel conformation of AMPA receptors, we did not take the commonly used approaches, such as structure-based inhibitor design or organic synthesis of small molecules. There has been no structural information available for sites of noncompetitive inhibitors on AMPA receptors. There has not been any type of structural templates, synthetic or natural products alike, which could have served as a template for developing a conformation-selective inhibitor of AMPA receptors. Because of these limitations, we decided to use an in vitro evolution approach, known as systematic evolution of ligands by exponential enrichment (SELEX), to identify potential RNA inhibitors or aptamers from an RNA library that comprised of $\sim 10^{15}$ randomized sequences (12-13). This approach enables one to identify a desired RNA molecule(s) with a defined property against the target without pre-existing templates (14) – a concept and practice different from organic synthesis, the most commonly used approach for developing inhibitors and drugs (15-16). RNA aptamers can fold into potentially useful three-dimensional structures, and can be identified using an in vitro elution approach to recognize virtually any target molecules as well as perform desired functions with high affinity and selectivity not found in nature (17). Using this in vitro evolution approach, we have indeed found an aptamer, described below, that potently and selectively inhibit AMPA receptors by targeting uniquely the open-channel conformation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Receptor Expression

The original cDNAs encoding rat GluA1, 2 and 3 AMPA receptors and GluK2 kainate receptor were kindly provided by Steve Heinemann. The GluA4 DNA plasmid was kindly provided by Peter Seeburg. The GluK1 plasmid was kindly provided by Geoffrey Swanson. The cDNAs of all three N-methyl-D-aspartic acid (NMDA) receptor subunits were kindly provided by John Woodward. All of the receptors were transiently expressed in human embryonic kidney (HEK-293S) cell. HEK-293S cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin in a 37 °C, 5% CO₂, humidified incubator. The DNA plasmids encoding green fluorescent protein and large T-antigen were cotransfected in HEK-293S cells (18). Transfected cells were used for recording 48 hour after transfection. For the in vitro selection, the transfected cells were harvested 48 hours after transfection, and the membrane fragment that contained the GluA2Q_{flip} receptor was prepared as described (19).

¹Abbreviations:

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CPM	counts per min
HEK-293 cells	human embryonic kidney cells
NMDA	N-methyl-D-aspartic acid
nt	nucleotide
SELEX	systematic evolution of ligands by exponential enrichment

In Vitro Selection

The preparation of the RNA library and the protocol of running the in vitro evolution selection were described previously (19) (Figure S1, Supporting Information). For binding in the initial round of selection, the RNA library with $\sim 10^{15}$ random sequences was dissolved in the extracellular buffer, which contained (in mM) 150 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4). The final concentration of membrane-bound receptor in the binding mix was 8 nM, as determined by [³H]AMPA binding. To keep the homomeric GluA2Q_{flip} ion channels at the open-channel conformation, the membrane-bound receptor was exposed to 1 mM (final concentration) of kainate for 5 min before mixing with the RNA library. The mixture of the RNA library and the receptor was incubated at 22 °C for 50 min for RNA binding to the receptor in the presence of 0.3 units/ μ l RNase inhibitor. For elution, we used 1 mM (final concentration) of GYKI 47409. The eluted RNAs were then subject to reverse transcription/PCR (Figure S1, Supporting Information). At the end of the 14th selection round, the DNA pools from rounds 12 and 14 were separately cloned into the pGEM-T easy vector (Invitrogen) for sequencing. By sequence comparison, the enriched sequences were identified (Figure 1A) and tested functionally, described below.

Homologous Competitive Binding Assay

The 5'-end ³²P-labeled aptamer was first prepared as described (19). Then, 1 μ l of 10 nM of ³²P-labeled aptamer was mixed with 2 μ g of yeast tRNA (Sigma) and a series of concentrations (i.e., 0-400 nM, final) of unlabeled (cold) aptamer. For receptor preparation, 4 femtomol of the membrane-bound GluA2Q_{flip} was suspended in the extracellular buffer with and without 1 mM of kainate. The final concentration for the receptor and for the hot aptamer, after mixing, was 0.4 nM and 0.1 nM, respectively. The mixture was incubated at 22 °C for 1 hr for binding. The mixture was loaded onto a pre-soaked 0.45 μ m nylon filter (VWR), which was then centrifuged at 4000 rpm for 5 min. The filter was washed twice with 400 μ l of the extracellular buffer. The radioactivity on the filter was quantified in a scintillation counter (Beckman LS6500). The analysis of the homologous competition binding data (20) is described in detail in Appendix.

Whole-Cell Current Recording

The procedure for whole-cell current recording to assay the inhibitory property of an RNA aptamer was previously described (19). The electrode for whole-cell recording had a resistance of ~ 3 M Ω , when filled with the electrode solution (in mM): 110 CsF, 30 CsCl, 4 NaCl, 0.5 CaCl₂, 5 EGTA, and 10 HEPES (pH 7.4 adjusted by CsOH). The extracellular buffer composition was provided under the "In Vitro Selection". For recording of the NMDA channels, the intracellular solution contained (in mM) 140 CsCl, 1 MgCl₂, 0.1 EGTA, and 10 HEPES (pH 7.2 adjusted by Mg(OH)₂), while the extracellular solution contained (in mM) 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 10 glucose and 5 HEPES (pH 7.2 adjusted by NaOH). In the extracellular buffer, 2 μ M of glycine was added (19). All reagents including aptamer were dissolved in the corresponding extracellular buffer and used. All RNA samples were transcribed and purified as described (19). The glutamate-induced whole-cell current was recorded using an Axopatch-200B amplifier at a cutoff frequency of 2-20 kHz by a built-in, eight-pole Bessel filter and digitized at 5-50 kHz sampling frequency using a Digidata 1322A from Axon Instruments (Molecular Devices, Sunnyvale, CA). The pClamp 8 software (Molecular Devices) was used for data acquisition. All whole-cell recordings were at -60 mV and 22 °C.

Laser-Pulse Photolysis Measurements

The laser-pulse photolysis technique was used to measure the channel-opening kinetics (21). Briefly, the caged glutamate (22) (In vitrogen, Carlsbad, CA) (or free glutamate) with or

without aptamer was dissolved in the extracellular buffer and applied to a cell using a flow device (19,23). In the laser-pulse photolysis measurement of channel opening, a single laser pulse at 355 nm with a pulse length of 8 ns was generated from a pulsed Q-switched Nd:YAG laser (Continuum, Santa Clara, CA). The pulse energy varied in the range of 200-800 μ J, measured at the end of an optical fiber (300 μ m core diameter) into which the laser was coupled. To calibrate the concentration of photolytically released glutamate, we applied two solutions of free glutamate with known concentrations to the same cell before and after a laser flash (24). The current amplitudes obtained from this calibration were compared with the amplitude from the laser measurement with reference to the dose-response relationship (21). The analysis of channel-opening kinetic data is described in detail in Appendix.

Statistical Data Analysis

Unless noted otherwise, each data point, such as A/A(I) or binding data point, was an average of at least three measurements (each of the whole-cell recording data was collected from at least three cells). Uncertainties reported refer to standard deviation from the mean. The significance of inhibition was evaluated by a one-sample two-tailed Student's *t* test with the assumption that $H_0: \mu = \mu_0 = 1$, 1 being the theoretical value of no inhibition and indicated by single ($P \leq 0.05$) or double ($P \leq 0.01$) blue asterisk sign. The significance of the difference between the open-channel and the closed-channel conformations was evaluated by a two-sample two-tailed Student's *t* test with the assumption that $H_0: \mu_1 = \mu_2$ and indicated by single ($P \leq 0.05$) or double ($P \leq 0.01$) red asterisk sign. Origin 7 was used for data analysis and plotting.

RESULTS

Design Strategy for Discovery of Aptamer Targeting the Open-Channel Conformation of AMPA Receptors

In this work, we chose to use the GluA2Q_{flip} AMPA receptor as the target of in vitro selection (Experimental Procedures and Figure S1, Supporting Information). GluA2 is one of the four AMPA receptor subunits, and can form homomeric, functional channel by itself, like any other AMPA receptor subunits (9). GluA2 is considered a key subunit that mediates excitotoxicity (25). The “flip” isoform of GluA2Q or GluA2Q_{flip}, generated by alternative splicing, is known to desensitize less rapidly than the “flop” isoform (5, 26). The edited or the Q isoform (i.e., glutamine at the glutamine/arginine or Q/R editing site) is calcium-permeable, whereas the R isoform is not (27). An abnormal expression of the Q isoform of GluA2 is linked to neurological disorders such as ALS (28).

To make it practically possible to apply an in vitro evolution approach to identifying aptamers against the open-channel conformation of AMPA receptors, we specifically designed the following experiments. First, we used a saturating agonist concentration to “titrate” the receptor population to maximize the fraction of the open-channel conformation of GluA2Q_{flip}. In other words, we wanted to present specifically the open-channel conformation of GluA2Q_{flip} as the target of the selection in anticipation of finding aptamers that would specifically recognize the open-channel conformation. Second, the open-channel conformation lasts no more than a millisecond or so (depending on the glutamate concentration) after glutamate binding (5), whereas the binding reaction between the receptor and RNA library requires at least 30 min to complete (19) (Experimental Procedures and Figure S1, Supporting Information). Thus, we had to “trap” the open-channel conformation long enough for the binding reaction. To do so, we decided to choose kainate as the agonist. Kainate is capable of producing a non-desensitizing current response with GluA2 after kainate binds to it, indicative of a persistent existence of the open-channel

conformation (29). Experimentally, we preincubated the cell membrane containing the GluA2Q_{flip} receptor with 1 mM kainate (i.e., this was a saturating concentration). Third, we used a noncompetitive inhibitor, i.e., GYKI 47409, to elute putative RNAs that might bind to the same site or mutually exclusive sites(s) (Figure S1, Supporting Information). GYKI 47409 is a 2,3-benzodiazepine derivative and has an inhibition constant (K_i) of ~3 μ M for the open-channel conformation of GluA2Q_{flip} or ~2-fold higher affinity than towards the closed-channel conformation (Pei and Niu, unpublished data). The overall design of our experiments was to specifically identify an aptamer that targeted the open-channel conformation by its binding to a noncompetitive site.

Identification of an RNA Aptamer that Inhibits AMAP Receptors

The GluA2Q_{flip} channels were transiently expressed in HEK-293S cells, and the membrane fragments harboring the entire functional receptors were used for in vitro selection (19). To suppress potentially hazardous enrichment of nonspecific RNAs bound to any other “targets”, such as lipids, we also carried out the negative selection as in rounds 5, 10 and 13, in a total of 14 selection cycles, in which plain HEK-293 cell membrane lacking only the GluA2Q_{flip} receptors was used to absorb these nonspecific RNAs. In contrast, the positive selection rounds involved the use of GYKI 47409, as mentioned before, to elute potentially useful RNAs. The eluted RNAs were amplified by RT-PCR, and an enriched RNA library was then transcribed for a new round of selection (Figure S1, Supporting Information). After 14 cycles, we identified some enriched sequences (Figure 1A). An enriched sequence was one with at least two copies in the entire sequence pool of 83 clones (i.e., 43 clones from round 12 and 40 clones from round 14). The putative inhibitory property of these enriched sequences was then functionally tested by the use of whole-cell current recording with GluA2Q_{flip} expressed in HEK-293 cells. Based on the whole-cell recording results (see representative traces in Figure 1B) or the ratio of the current amplitudes in the absence and presence of an aptamer, A/A(I) (shown on the right column in Figure 1A), we concluded that AG1407, the most enriched sequence, was one of the most potent inhibitors. A further test of AG1407 at the same aptamer concentration but with increasing glutamate concentrations showed that AG1407 inhibited the open-channel conformation of GluA2Q_{flip} (Figure S2, Supporting Information).

Identification of the Minimal, Functional Aptamer Sequence

Next we systematically truncated aptamer AG1407 in order to identify the minimal, yet functional sequence. Guided by the secondary structure prediction using the Mfold program (30), we constructed and functionally tested shorter versions of AG1407 (Figure 2A). Based on A/A(I) value (shown in red color at the bottom of each predicted structure in Figure 2A), we found the 56-nucleotide (nt) version of AG1407, termed as AG56, was the functional aptamer with the minimal length of sequence. In contrast, either shortening the three-way junction by deleting UUGUGA sequence (i.e., the 46 nt RNA) or removing the bulge at the U50 position (i.e., 45 nt RNA) or truncating the base-paired stem in the first stem-loop region (i.e., 48 nt RNA) (Figure 2A) resulted in nonfunctional RNAs. Therefore these structural elements are essential in the folding of AG56 as a functional aptamer. Consequently we used AG56 as the minimal aptamer for all of the functional characterizations described below.

Functional Characterization of Aptamer AG56 by Whole-Cell Recording

AG56 was functionally characterized in a series of experiments. First, like its predecessor sequence AG1407 (Figure S2, Supporting Information), AG56 selectively inhibited the open-channel, but not the closed-channel, conformation of GluA2Q_{flip} (Figure 2B, left panel). Furthermore AG56 similarly inhibited the open-channel conformation of all other AMPA receptor subunits, i.e., GluA1, 3 and 4, although the inhibitory effect of AG56 on

GluA4 was weak (Figure 2B left panel, and Figure S3, Supporting Information). Yet, AG56 had no inhibitory effect on any of the closed-channel conformations (Figure 2B left panel). AG56 did not affect either the kainate receptor channels (i.e., GluK1 and GluK2) or the NMDA receptor channels (i.e., GluN1A/2A and GluN1A/2B) (Figure 2B right panel). It should be noted that GluN1A/2A and GluN1A/2B are two dominant NMDA receptor complexes in vivo (31) and neither GluN1A nor GluN2A or GluN2B can form a functional channel by itself (32). These results thus suggest that AG56 is an AMPA receptor-subtype selective inhibitor by targeting the open-channel conformations, and AG56 is without any unwanted, cross activity against other glutamate receptor subtypes.

Mechanism of Inhibition of AG56 on GluA2Q_{flip}: Homologous Binding Studies

We further elucidated the mechanism of action of AG56 on the GluA2Q_{flip} receptor channel expressed in HEK-293 cells. In this study, we first determined the inhibition constant of AG56 to be $0.95 \pm 0.20 \mu\text{M}$ (the solid line in Figure 3A) for the open-channel conformation of GluA2Q_{flip} at 3 mM glutamate concentration where almost all of the channels were in the open-channel conformation (this was because the EC_{50} value of GluA2Q_{flip} with glutamate was 1.3 mM and the channel-opening probability of GluA2Q_{flip} was near unity (21)). In contrast, AG56 did not inhibit the closed-channel conformation of GluA2Q_{flip}, as verified by a series of aptamer concentrations (Figure 3A) but at 100 μM glutamate concentration where most of the receptors was in the closed-channel conformation (21). This result could be explained by a noncompetitive mechanism by which AG56 bound to a regulatory site or noncompetitive site, and such a site was accessible from both the closed-channel and the open-channel states or conformations; yet only the interaction of the aptamer with the open-channel conformation resulted in inhibition. Alternatively, this result could be explained by an uncompetitive mechanism, such as an open-channel blockade model, by which AG56 would only inhibit the open-channel conformation, because the uncompetitive site would only be accessible through the open-channel conformation (33) (see the two mechanisms in Appendix). To differentiate these two mechanisms, we first carried out a homologous competition binding assay (20) and found that AG56 not only bound to the closed-channel conformation (i.e., the unliganded, closed-channel receptor form) but did so with an affinity, i.e., $K_d = 68 \pm 40 \text{ nM}$ (Figure 3B left panel) similar to that for the open-channel conformation, i.e., $K_d = 80 \pm 23 \text{ nM}$ (Figure 3B right panel). This result was consistent with a noncompetitive mechanism, based on the fact that AG56 was found to bind to the closed-channel conformation in addition to its binding to the open-channel conformation. This result, however, was inconsistent with the uncompetitive mechanism.

Mechanism of Inhibition of AG56 on GluA2Q_{flip}: A Laser-Pulse Photolysis Measurement of the Effect of AG56 on the Channel-Opening Rate Process

We further characterized the mechanism of inhibition of AG56 on the channel-opening kinetic process of GluA2Q_{flip}. Using a laser-pulse photolysis technique, together with a photolabile precursor of glutamate or caged glutamate, which provided a time resolution of ~30 microsecond (22), we specifically measured the effect of AG56 on both the channel-closing (k_{cl}) and the channel-opening (k_{op}) rate constants (23) (Figure 3C left and middle panels, respectively). This experiment enabled us to simultaneously follow not only the rate of channel opening but also the current amplitude, prior to channel desensitization (23) (Figure 3C, left and middle panels). The magnitude of k_{cl} reflects the lifetime (τ) of the open channel (i.e., $\tau = 1/k_{cl}$) and the effect of an inhibitor on k_{cl} thus reveals whether or not it inhibits the open-channel conformation (23). In contrast, k_{op} reflects the closed-channel conformation and the effect on k_{op} reveals whether the inhibitor inhibits the closed-channel conformation (23) (see the rate equations and quantitative treatment of the rate data in Appendix). Experimentally, at a low glutamate concentration (i.e., 100 μM photolytically released glutamate) where k_{cl} was measured (23), AG56 inhibited the rate of channel

closing, consistent with a noncompetitive mechanism by which it inhibited the open-channel conformation. Yet, AG56 did not affect the current amplitude (Figure 3C left panel). This was not surprising because the amplitude observed at this low glutamate concentration (i.e., 100 μ M photolytically released glutamate) was dominated by the closed-channel receptor population (notice this was consistent with the amplitude measurement shown as the red dashed line in Figure 3A).

However, when the concentration of glutamate increased and k_{op} became measurable (23) (see Appendix), AG56 did not inhibit k_{op} (Figure 3C middle and right panels). This result suggested that the inhibition of the rate by AG56 could be completely ascribed to the inhibition of k_{cl} by AG56 such that the difference between the observed rate constant of channel opening or Δk_{obs} in the absence and presence of AG56 at the same AG56 concentration was invariant in spite of increasing glutamate concentration (Figure 3C right panel, and see the mechanistic treatment of the rate data, specifically equ. 9, in Appendix). In other words, the fact that Δk_{obs} remained the same (or $\Delta k_{obs} = \text{constant}$), verified in a series of increasing glutamate concentrations, was entirely consistent with the prediction by equ. 9 (see additional explanation about equ. 9 in Appendix). The lack of an inhibitory effect of AG56 on k_{op} (Figure 3C middle and right panels) further demonstrated that AG56 did not inhibit the closed-channel conformation.

On the other hand, AG56 reduced the current amplitude at a higher glutamate concentration (see the difference in peak current amplitudes between the red and blue traces in Figure 3C middle panel; and see an additional trace in Figure S4, Supporting Information, for a higher current amplitude inhibition at a higher glutamate and inhibitor concentration). Again, this was expected because the current amplitude at a higher glutamate concentration began to reflect more on the open-channel receptor population. Furthermore, the effect of AG56 on the current amplitude from the rate measurement (Figure 3C middle panel, and Figure S4, Supporting Information) was entirely consistent with the amplitude measurement using a rapid solution flow method (Figure 3A). Taken together, the results from the binding site/affinity assessment (Figure 3B) and the chemical kinetic characterization of the effect of AG56 on both k_{cl} and k_{op} (Figure 3C) as well as the amplitude measurement (Figure 3A) are consistent only with AG56 being a noncompetitive inhibitor selective to the open-channel receptor conformation. Conversely, an uncompetitive mode of action is inconsistent with our data, i.e., AG56 would only bind to the open-channel conformation, but not the closed-channel conformation. A competitive mode of action is also inconsistent with our data, i.e., AG56 would only be effective as an inhibitor at a low ligand concentration and would inhibit k_{op} , but not k_{cl} .

DISCUSSION

In this study, we have described the discovery of a novel inhibitor of AMAP receptors, i.e., aptamer AG56, through an in vitro evolution selection. Using a combination of equilibrium binding and rapid chemical kinetic measurements, we have characterized the mechanism of action of AG56 and have concluded that AG56 inhibits the GluA2Q_{flip} receptor noncompetitively. This conclusion is not surprising, because 2,3-benzodiazepine compounds like the one we used (i.e., GYKI 47409) in the in vitro selection are known as noncompetitive inhibitors. Aptamer AG1407, the predecessor of AG56, which was eluted from the GluA2Q_{flip} receptor by GYKI 47409, was supposedly bound to the same noncompetitive site. In addition, because the open-channel conformation of GluA2Q_{flip}, rather than the closed-channel conformation or a conformation mixture, was exclusively presented as the target of the selection, AG56 was selected, as expected, to specifically recognize and inhibit the open-channel conformation of GluA2Q_{flip}.

The results we have obtained from this study also provide insights into the structural similarity of the open-channel conformations among AMPA receptor subunits. As anticipated, AG56 showed the selectivity towards the open-channel conformation of the GluA2 AMPA receptor subunit because GluA2, precisely the open-channel conformation of the GluA2, was exclusively presented as the target in the in vitro evolution selection. Interestingly, however, AG56 showed likewise the open-channel conformation selectivity towards the rest of the AMPA receptor subunits, despite the fact that these AMPA receptor subunits had never been presented for evolution selection. A successful in vitro selection of an aptamer from its target is based, at least in part, on “fitness” or the molecular recognition between the aptamer and its target (14). Our result therefore suggests that there is a considerable structural similarity among AMPA receptor subunits at the level of the open-channel conformation. However, whether there is the same level of structural similarity in the closed-channel conformation among all of the AMPA receptor subunits awaits future studies. This question is important because the answer may well inform what would be a more effective conformation for the development of AMPA receptor subunit-selective inhibitors. Such an inhibitor does not exist today, and developing such an inhibitor will give us additional ability of controlling the function of an AMPA receptor one subunit at a time. If our hypothesis is plausible, the open-channel conformation will be a structural platform that can be used for developing nanomolar affinity noncompetitive inhibitors for the AMPA receptor subtype; however, it might be difficult to find inhibitors selective to single AMPA receptor subunit using this platform because of a considerable structural similarity at the level of the open-channel receptor conformation of all AMPA receptors.

The fact that AG56 possesses the unique selectivity towards the open-channel receptor conformation of all AMPA receptors without any inhibitory effect on the closed-channel conformation suggests that this noncompetitive inhibitor may be useful for allowing us to control the AMPA receptor activity in vivo more tightly with minimal or none off-target activity. This is because the inhibition of AMPA receptors by AG56 is based on a greater molecular recognition between AG56 and the specific conformational state of AMPA receptors, rather than a promiscuous recognition of a mixture of functional states. The fact that AG56 targets the open-channel receptor conformation further suggests that the apparent inhibition potency of this noncompetitive aptamer is stronger when the agonist concentration is higher, such as under excitotoxic conditions (see the treatment of amplitude data in Appendix or a graphic illustration of this notion in Figure 3A). As a comparison, a competitive inhibitor loses its inhibitory potency when agonist concentration increases (because they compete to the same site) (19). Therefore the discovery of an open-channel conformation selective inhibitor AG56 not only represents our continuing effort for developing a “toolbox” of aptamer inhibitors that include competitive (19) and now noncompetitive inhibitors, but also gives us additional ability to regulate AMPA receptor activities in a wide range of glutamate concentrations.

Our results have demonstrated that using the in vitro evolution method, we can successfully isolate a potent noncompetitive RNA inhibitor from a random sequence library. However, the use of this approach to evolve aptamers against a desired target requires target preparation, particular if the target is a membrane protein. Previously, we reported that glutamate ion channels can be expressed in HEK-293 cells and the membrane fragments harboring total, functional receptors can be prepared for a successful in vitro selection of competitive aptamers (19). In the present study, we have shown that such a method is also successful in evolving noncompetitive inhibitors. In a technically simpler and more conventional approach, a soluble portion of a membrane protein can be used for in vitro selection, and such a water-soluble, partial AMPA receptor construct, which comprises of the extracellular binding domain of the AMPA receptors, known as S1S2 protein, has been available (34). However, we found previously that GYKI compounds do not bind to this

water-soluble S1S2 mini-receptor (23). Therefore, the target preparation, i.e., the use of the entire functional receptor or GluA2Q_{flip} embedded in lipid membrane, is a critical component of our success in finding a noncompetitive aptamer, not just the use of an in vitro selection approach.

How are the properties of AG56 compared with those of small molecule inhibitors? First, the high conformation selectivity of AG56 as an AMPA receptor inhibitor is unique. Second, AG56 is more potent than the GYKI 47409, the elution agent. In fact, the inhibitory potency of AG56 rivals any existing noncompetitive inhibitors documented thus far. Third, small molecule inhibitors of AMPA receptors, prepared by synthetic chemistry, such as quinoxalines, and 2,3-benzodiazepine compounds, generally have limited water solubility, which so often plagues the clinical usefulness of these compounds (35). In contrast, RNA aptamers are naturally water soluble. Therefore, AG56 represents a water-soluble, highly potent, and highly selective inhibitor of AMPA receptors. It does not have any undesirable cross activity on either kainate or NMDA receptors.

The data we presented here demonstrate that the open-channel conformation, which lasts in less than a few ms in general once glutamate is bound, can be potently and selectively inhibited by drug-like molecules in the sub-nanomolar concentration range. As an RNA molecule, AG56 is readily amenable to structural modifications and large-scale synthesis or transcription for further development into a potent, selective drug candidate and/or biochemical probe targeting AMPA receptors. For instance, AG56 can be chemically modified to become a covalent labeling agent for mapping the noncompetitive site of action (36). To date, there is no structural information available for any noncompetitive sites on any AMPA receptors. Furthermore, AG56 can be used as the template of chemical modifications to make it ribonuclease-resistant or biostable (37). A bio-stable aptamer can be practically tested in vivo as a viable pharmacological and therapeutic agent for the diagnosis and potential treatment of neurological disorders and diseases. Finally, because AG56 is a water-soluble, noncompetitive inhibitor, our finding represents a major step forward in exploration of making alternative agents that target excitotoxicity involving excessive AMPA receptor activity.

In conclusion, using an in vitro evolution approach and a random RNA sequence library, we have shown that we can successfully “breed” a new RNA molecule de novo that are capable of noncompetitively inhibiting AMPA receptor activity with nanomolar potency and unique conformation selectivity. Our approach is the alternative to traditional strategies of making noncompetitive inhibitors such as by synthetic chemistry. The discovery of AG56 offers promising opportunities to exploit the use of this aptamer both as a mechanistic probe in structure-function studies of AMPA receptors and as a lead molecule in development of potential therapeutic agents for a number of neurological disorders and diseases. In this context, the unique selectivity of AG56 towards the open-channel receptor conformation of AMPA receptors without any inhibitory effect on the closed-channel conformation offers a possibility of controlling the receptor activity in vivo more tightly with minimal or none off-target activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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APPENDIX

Homologous Competitive Binding Assay

The non-specific binding was estimated by equ.1. The specific binding was calculated as the difference between the total binding count or counts per min (CPM) and the estimated non-specific CPM. The specific binding was also normalized to percentage based on the CPM value without the non-labeled AG56. Assuming a one-site binding model, the K_d of AG56 bound to GluA2Q_{flip} was estimated by fitting the binding data to equ. 1 (20)

$$Y = \frac{B_{\max} \times [\text{Hot}]}{[\text{Hot}] + [\text{Cold}] + K_d} + \text{NSB} \quad \text{equ. 1}$$

where [Hot]/[Cold] are the concentrations of the unbound, hot AG56/unlabeled or cold AG56, respectively; NSB represents non-specific binding.

Kinetic Data Analysis: Mechanism of Channel opening

Before we describe the kinetic investigation of the mechanism of inhibition, we first introduce the kinetic characterization of channel-opening rate process. Using the laser-pulse photolysis technique, we previously determined the rate constants for the GluA2Q_{flip} channel opening (21), based on a general mechanism of channel opening shown below.

A represents the active form of the receptor, L , the ligand, AL_n the closed-channel forms, and $\bar{A}L_n$ the open-channel form, K_1 the intrinsic dissociation constant of activating ligand and Φ the channel opening equilibrium constant ($\Phi^{-1} = k_{cl}/k_{op}$). Based on this mechanism and the assumption that the ligand-binding rate was fast as compared to the channel opening, the observed rate constant of channel opening or k_{obs} can be formulated as in equ. 2.

$$k_{obs} = k_{cl} + k_{op} \left(\frac{L}{K_1 + L} \right)^n \quad \text{equ. 2}$$

$$I_t = I_{\max} [1 - \exp(-k_{obs}t)] \quad \text{equ. 3}$$

In equ. 2, k_{cl} and k_{op} are the channel-closing and channel-opening rate constant, respectively. The n is the number of the ligand molecules that bind to the receptor to open the channel (i.e., $n = 1-4$). $I_{\max}$ is the maximum current amplitude, and I_t is the current amplitude at time t . Our previous studies of AMPA receptors, including a mutant AMPA receptor, for their channel-opening kinetic mechanisms led us to conclude that binding of two glutamate molecules per receptor (i.e., $n = 2$) was sufficient to open the channel (38). Using the laser-pulse photolysis technique, we previously determined the k_{op} of $(8.0 \pm 0.49) \times 10^4 \text{ s}^{-1}$ and the k_{cl} of $(2.6 \pm 0.20) \times 10^3 \text{ s}^{-1}$, respectively, for the channel-opening kinetic constants of the GluA2Q_{flip} receptor (21).

Kinetic Data Analysis: Mechanism of Inhibition

The mechanism of inhibition was characterized by measuring the effect of AG56 on the channel-opening rate process (33,39). First let us assume that an inhibitor binds only to the open-channel conformation and then inhibits it (Mechanism 1, uncompetitive mechanism of inhibition or open-channel blockade). Alternatively, an inhibitor binds to both the closed- and open-channel states through a regulatory site (Mechanism 2, noncompetitive mechanism). The relationship between k_{obs} the molar concentration of the ligand (glutamate), L , and the inhibitor, I , can be written according to the individual mechanism, i.e., equ. 4 for mechanism 1, and equ. 5 for mechanism 2.

Mechanism 1

$$k_{obs} = k_{op} \left(\frac{L}{L + K_1} \right)^n + k_{cl} \left(\frac{\overline{K_I}}{\overline{K_I} + I} \right)$$

equ. 4

Mechanism 2

$$k_{obs} = k_{op} \left(\frac{L}{L + K_I} \right)^n \left(\frac{K_I}{K_I + I} \right) + k_{cl} \left(\frac{\overline{K}_I}{\overline{K}_I + I} \right)$$

equ. 5

In deriving these equations, one binding site for inhibitor per receptor molecule is assumed. At low concentrations of glutamate ($L \ll K_I$), k_{obs} reflects the channel-closing rate constant since the contribution of the k_{op} portion in equ. 4 or 5 to the overall rate, k_{obs} , is negligible. Under this condition, the effect of the inhibitor on the k_{cl} can be measured (33). Specifically, for both Mechanisms 1 and 2, the effects of the inhibitor on k_{cl} are the same, and can be obtained by using equ. 6 below, which can be derived from either equ. 4 or 5. We have previously shown that the k_{obs} value obtained at 100 μ M glutamate concentration for GluA2Q_{flip} reflects the k_{cl} (21).

$$k_{obs} = k_{cl} \left(\frac{\overline{K}_I}{\overline{K}_I + I} \right)$$

equ. 6

In the experiment described in this study, the fact that we observed the inhibition of AG56 on k_{cl} suggested that AG56 inhibited the open-channel conformation of the GluA2Q_{flip} receptor (see Figure 3c, left panel and the legend). Not surprisingly, AG56 was also found to bind to the open-channel conformation (see Figure 3B, right panel).

The effect of an inhibitor on k_{op} is obtained at a high glutamate concentration (where $k_{obs} > k_{cl}$). For Mechanism 1 or uncompetitive mechanism, the inhibitor *does not* affect k_{op} (equ. 7). The lack of inhibition of the closed-channel conformation, manifested by the lack of effect on k_{op} , is apparently due to the fact that the inhibitor does not even bind to the closed-channel conformation. In our case here, however, AG56 did bind to the closed-channel conformation, and this result was inconsistent with the uncompetitive mechanism of inhibition, described above.

$$k_{obs} - k_{cl} \left(\frac{\overline{K}_I}{\overline{K}_I + I} \right) = k_{op} \left(\frac{L}{L + K_I} \right)^n$$

equ. 7

For Mechanism 2 or a noncompetitive mechanism of inhibition, the inhibitor will affect k_{op} additionally (equ. 8), if $K_I < I$, I being the molar concentration used to measure the K_I value. It should be emphasized that the presence of an inhibitory effect of a noncompetitive inhibitor is due to the fact that the inhibitor must first bind to the closed-channel conformation, as in Mechanism 2.

$$k_{obs} - k_{cl} \left(\frac{\bar{K}_I}{\bar{K}_I + I} \right) = k_{op} \left(\frac{L}{L + K_1} \right)^n \left(\frac{K_I}{K_I + I} \right) \quad \text{equ. 8}$$

If, however, the inhibitory effect on the closed-channel conformation is so weak (or $K_I \gg I$), the inhibitor literally no longer inhibits the closed-channel conformation (but still binds to the site). Under this circumstance, the difference in k_{obs} at a defined ligand concentration in the absence (as illustrated in equ. 2, which we termed as k_{obs}) and presence of a noncompetitive inhibitor (as illustrated in equ. 5, which we termed as k_{obs}') will be independent of the ligand concentration, as is shown in equ. 9:

$$\begin{aligned} \Delta k_{obs} = k_{obs} - k_{obs}' &= \left[k_{op} \left(\frac{L}{L + K_1} \right)^n + k_{cl} \right] - \left[k_{op} \left(\frac{L}{L + K_1} \right)^n \left(\frac{K_I}{K_I + I} \right) + k_{cl} \left(\frac{\bar{K}_I}{\bar{K}_I + I} \right) \right] \\ \Delta k_{obs} &= k_{cl} - k_{cl} \left(\frac{\bar{K}_I}{\bar{K}_I + I} \right) = \Delta k_{cl} \end{aligned} \quad \text{equ. 9}$$

In deriving equ. 9, we assumed that $K_I \gg I$; thus the k_{op} portions are canceled off. Based on equ. 9, a plot of $\Delta k_{obs} = k_{obs} - k_{obs}' = \Delta k_{cl}$ vs. glutamate concentration would be invariant under the same inhibitor concentration despite the fact that glutamate concentration was varied. This was exactly the case with AG56 (see Figure 3C). Our results suggested that AG56 was bound to the closed-channel conformation (Figure 3B, left panel) as a noncompetitive inhibitor, but the binding to the closed-channel conformation of GluA2Q_{flip} was not efficacious or not inhibitory.

Amplitude Data Analysis

The amplitude ratio, $A/A(I)$, can be used to independently obtain inhibition constants. The experimental design of using current amplitude to determine the inhibition constant for both the open-channel and the closed-channel conformations requires varying concentration of glutamate, as shown in equ. 10a and 10b. Equ. 10a and 10b are derived based on one inhibitor binding to the receptor. $K_{I,app}$ is the apparent inhibition constant for the inhibitor; other terms have been defined previously.

$$\frac{A}{A(I)} = 1 + I \frac{\bar{A}L_2}{K_{I,app}} \quad \text{equ. 10a}$$

$$\left(\bar{A}L_2 \right) = \frac{\bar{A}L_2}{A + AL + AL_2 + \bar{A}L_2} = \frac{L^2}{L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi} \quad \text{equ. 10b}$$

Specifically in our experiments, at low glutamate concentrations (i.e., $L \ll K_I$), the majority of the receptors in a receptor population were in the closed-channel conformation, since we measured the macroscopic current response or the response from an ensemble of receptors expressed in a single HEK-293 cell. Under this condition, the inhibition constant for the closed-channel conformation was determined from the ratio of the amplitude according to equ. 10a and 10b. Likewise, at a saturating ligand concentration (i.e., $L \gg K_I$), the majority of the receptors were in the open-channel state. Consequently, the inhibition constant associated with the open-channel conformation was measured. The basis of using the two ligand concentrations that corresponded to ~4% and ~96% fraction of the open-channel receptor form (21) to determine the corresponding inhibition constant was a putative difference in inhibition constant between the closed-channel and the open-channel conformation. At those low and high ligand concentrations (21), the apparent inhibition constants obtained were considered pertinent to the closed-channel and the open-channel conformations, respectively (23).

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Figure 1.

The enriched RNA sequences and their biological functions. (A) The seven enriched sequences are shown with their names on the left and the copy number on the right, which represents the number of appearances of the same sequence in the entire sequence pool (83 sequences – see the text). The variable sequence region (N50) and the selected sequences are shown in green color, whereas the 5' and 3' constant regions are displayed at the bottom. The putative inhibition of GluA2Q_{flip}, the SELEX target, by these RNAs, as tested by whole-cell recording, is shown, on the right, as the ratio of the whole-cell current amplitude in the absence and presence of 500 nM of aptamer or A/A(I) at 3 mM glutamate. (B) Representative traces of the whole-cell current response of GluA2Q_{flip} to 3 mM of glutamate in the presence and presence of 500 nM of AG1407. The current was recorded at –60 mV, pH 7.4 and room temperature with the same HEK-293 cell expressing GluA2Q_{flip}.

Figure 2.

The minimal, functional sequence of the aptamer and the AMPA-receptor subtype selectivity. (A) In truncating the sequence of AG1407 to identify the minimal, functional 56-nt sequence or AG56, only the most stable secondary structure of each sequence, with the free energy listed below that structure, was considered, based on the prediction by Mfold. The black and brown colored letters represent the constant and variable sequence regions, respectively. A “scissor” indicates where a particular sequence was cut, and the green letters represent the cut-off nucleotides. The inhibitory function, shown as A/A(I) value, is listed at the bottom of each truncated structure. (B) By whole-cell current recording assay, AG56 selectively inhibited the open-channel conformation of all AMPA receptor subunits (left panel) (see further explanation and statistical analysis in Figure S3, Supporting Information). Yet, AG56 did not affect GluK1Q and GluK2Q, two representative kainate receptor channels, nor GluN1A/2A and GluN1A/2B NMDA receptor channels. For each of the receptor types tested, the glutamate concentration was chosen to be equivalent to ~4% and ~95% fraction of the open channels. Specifically, the glutamate concentration was 0.04 mM (for the closed-channel conformation)/3 mM (for the open-channel conformation) of GluA1_{flip}, 0.1 mM/3 mM for GluA2Q_{flip}, GluA3_{flip}, and GluA4_{flip}, as well as 0.04 mM/3 mM for GluK1 and GluK2Q.

Figure 3.

Characterization of inhibition constant, binding affinity and mechanism of action of AG56 with GluA2Q_{flip} expressed in HEK-293 cells. (A) By whole-cell current recording assay, AG56 inhibited the open-channel (measured at 3 mM of glutamate concentration), but not the closed-channel (measured at 0.1 mM of glutamate concentration) conformation of GluA2Q_{flip} (the dotted line indicates no inhibition or $A/A(I) = 1$). The inhibition constant, K_I , for the open-channel conformation of GluA2Q_{flip} by AG56 was determined to be $0.95 \pm 0.20 \mu\text{M}$ (i.e., the upper solid line). (B) The homologous competition binding of AG56 to GluA2Q_{flip} receptor was plotted for both unliganded, closed-channel form (solid circle) and open-channel form (hollow circle). The binding constant, K_d , of AG56 to the closed-channel (left panel) and the open-channel (right panel) forms of GluA2Q_{flip} was determined to be $68 \pm 40 \text{ nM}$ and $80 \pm 23 \text{ nM}$, respectively, based on triplicate data sets. The binding constant was calculated using eq. 1 in Experimental Procedures. (C) The laser-pulse photolysis measurement of the effect of AG56 on the channel-closing rate constant or k_{cl} (left panel) and channel-opening rate constant or k_{op} (middle panel) with GluA2Q_{flip} expressed in HEK-293 cells. Specifically, at 100 μM photolytically released glutamate concentration, the k_{obs} value, which reflected k_{cl} , was decreased from $2,200 \text{ s}^{-1}$ (control or $-0.5 \mu\text{M}$ AG56, black trace, left panel) to $1,600 \text{ s}^{-1}$ ($+0.5 \mu\text{M}$ AG56, red trace, left panel). At 340 μM photolytically released glutamate concentration, the k_{obs} value, which reflected k_{op} (middle panel), was $5,128 \text{ s}^{-1}$ and $4,405 \text{ s}^{-1}$ in the absence and presence of $0.5 \mu\text{M}$ AG56. The difference, however, or $\Delta k_{obs} = k_{obs} - k_{obs}' = \Delta k_{cl}$ was invariant even when glutamate concentration increased (the right panel). Here $\Delta k_{cl} = k_{cl} - k_{cl}'$ where k_{cl}' is the inhibited k_{cl} value and the k_{cl} is the channel-closing rate constant without AG56 (see equ. 9 in Appendix). Each data point represents at least one measurement from a single cell where k_{obs} is the control rate constant and k_{obs}' is the rate constant in the presence of $0.5 \mu\text{M}$ AG56.