Published in final edited form as:

Brain Res. 2007 October 26; 1177: 1–8.

Factors affecting guanine nucleotide binding to rat AMPA receptors

Kyle Montgomery, Erika Suzuki, Markus Kessler, and Amy C. Arai

Dept of Pharmacology, Southern Illinois University School of Medicine, Springfield IL 62702, USA

Abstract

Glutamate receptors are competitively inhibited by guanine nucleotides. Insight into the physiological function of this inhibition would be greatly advanced if nucleotide binding could be eliminated through mutations without altering other aspects of receptor function, or if compounds were discovered that selectively prevent nucleotide binding. It was previously reported that a lysine in the chick kainate binding protein (cKBP) is specifically involved in guanine nucleotide binding. In the present study we mutated the equivalent lysine in the rat AMPA receptor subunit GluR1 flip to alanine (K445A) and assessed changes in nucleotide affinity from the displacement of [³H] fluorowillardiine. As in the cKBP, the affinity for nucleotides was greatly reduced while the binding affinity for agonists remained unchanged. The reduction in affinity was largest for GTP (factor of 5.8) and GDP (4.4) and minor for GMP and guanosine. This suggests that K445 is involved in stabilizing the second phosphate of the nucleotide. Given that bulkier analogs like GDP-fucose are also accommodated at this site, it seems likely that nucleotides bind in such a way that their phosphates project out of the cleft. In excised-patch recordings using short pulses of glutamate, the K445A mutation increased the EC₅₀ for the peak response 1.8 fold and accelerated desensitization and deactivation. This indicates that the effects of this mutation are not as specific as previously suggested. Efforts to selectively eliminate inhibition by nucleotides may therefore depend on mapping out further the docking site. In a first attempt using point mutations we ruled out several amino acids around the cleft as being involved in nucleotide binding. Also, the AMPA receptor modulator PPNDs which competitively inhibits nucleotide binding to purinergic receptors did not affect nucleotide inhibition, suggesting that there are major differences in the topography between purinergic and glutamate receptors. Thus new approaches, including crystallography, may be called for to identify residues uniquely involved in nucleotide binding.

Keywords

AMPA receptor subunits; mutation; PPNDs; suramin; GDP-fucose; GDP-mannose

INTRODUCTION

One of the perplexing and least understood properties of ionotropic glutamate receptors is their inhibition by nucleotides. NMDA, AMPA and kainate receptors (Monahan et al., 1988; Baron et al., 1989; Gorodinsky et al., 1993; Dev et al., 1996), as well as the structurally related kainate

Address for correspondence: Markus Kessler, Dept of Pharmacology, Southern Illinois University School of Medicine, 801 N. Rutledge, P.O.Box 19629, Springfield IL 62794-9629, 217-545-2204, 217-545-0145 (FAX), mkessler@siumed.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

binding proteins in fish, frogs and birds (Willard et al., 1991; Willard and Oswald 1992; Paas et al., 1996) are inhibited by guanine nucleotides, and NMDA receptors containing the NR2B subunit also bind adenine nucleotides (Ortinou et al., 2003). The phylogenetically early appearance and pervasiveness of this inhibition suggests that it serves an essential function. One proposition has been that nucleotides released from neurons during system failure, such as during ischemia, could reduce glutamate receptors activation and thereby protect against excitotoxic damage (Morciano et al., 2004). However, whether such protection constitutes sufficient grounds for evolutionary selection remains unclear. Interestingly, it has recently been shown that GTP is taken up into synaptic vesicles (Santos et al., 2006). Nucleotides may thus be co-released with glutamate and prolonged trains of synaptic activity could accordingly produce nucleotide concentrations high enough to attenuate synaptic responses. Inhibition by guanine nucleotides - like the more general inhibition of transmission by adenosine - may thus provide a feedback mechanism which continuously adjusts the efficacy of synaptic transmission. The question is again, however, whether this synaptic modulation is of such fundamental nature that it was selected for and conserved throughout evolution. A third possibility might be that glutamate receptors passing through the endoplasmic reticulum and Golgi stacks are at risk of being activated by glutamate diffusing into the lumen of these membranes which then could compromise the ability of these organelles to retain and regulate cytoplasmic calcium (Pinton et al., 1998). Nucleotide inhibition of glutamate receptors might in this case have co-evolved with glutamate activation as an intrinsic mechanism to minimize this 'endo-excitotoxicity'.

Studies concerning the function of nucleotide inhibition would be greatly facilitated if nucleotide binding could be eliminated without causing changes in agonist affinity and other aspects of receptor function. Interestingly, Paas et al. (1996) found that mutating a lysine residue to alanine in the chick kainate binding protein (cKBP) had precisely these effects. On the other hand, a physiological study by Li et al. (1995) found that changes in the corresponding lysine in rat AMPA receptors also reduced the EC_{50} for glutamate and AMPA when measuring steady-state currents in oocytes. In the latter study, however, lysine was mutated to glutamine or glutamate instead of alanine. It has thus been unclear if the differences in the findings between these two studies were due to the receptor subtype, the choice of the agonist, the nature of the mutation, or the experimental parameter under consideration. For the present study we therefore introduced the 'lysine to alanine' mutation into the rat AMPA receptor subunit GluR1 and examined if the proposition of Paas et al that guanine nucleotide affinity is selectively modulated also applies to rat AMPA receptors. Additional tests were conducted with the intent to map further the interaction between guanine nucleotides and AMPA receptors, as well as between guanine nucleotides and purinergic receptor antagonists that have recently been discovered to modulate AMPA receptors.

RESULTS

Because AMPA receptors have relatively low affinity for guanine nucleotides (Dev et al., 1996), affinities for the latter were determined by measuring the inhibition of the binding of the glutamate analog fluorowillardine (FW). In agreement with other studies, agonist binding to GluR1 was fully inhibited by guanosine nucleotides (figure 1 and table 1). In wildtype receptors, the affinity constants for GMP and GDP were similar (229 and 215 μ M, respectively) while GTP had a slightly lower affinity (311 μ M, $p=0.002$ versus GDP). Guanosine had a greatly reduced affinity (1.2 mM), indicating that the first phosphate group is of particular importance for the binding of nucleotides. The binding affinity varied less than three-fold across subunits, with GluR2 and 3 having the highest affinity, and it was similar in recombinantly expressed and native brain receptors (table 2).

Mutant receptors in which the positively charged lysine 445 had been replaced by an alanine (R1i-K445A; all numbering is given for the mature protein) exhibited significant reductions in the affinity for guanine nucleotides (figure 1C, table 1). Interestingly, the magnitude of this reduction depended greatly on the number of phosphates in the nucleotide. The loss in affinity was largest (5.8 fold) for GTP, about four-fold for GDP and less than two-fold for GMP. No change was seen in the affinity for guanosine. Importantly, the binding affinity for [³H] fluorowillardine was not significantly different between wildtype and mutant receptors (7.1 ± 0.6 and 5.9 ± 0.6 nM, respectively; $p = 0.215$; figure 1A), and virtually identical affinities were also obtained with [³H]AMPA as ligand (not shown). However, maximum binding ($B_{\max}$), which reflects the number of functional receptors, was about 50% lower in transfections with R1i-K445A.

Physiological recordings were conducted to assess if the K445A mutation influences basic response properties and if it alters guanine nucleotide inhibition as predicted by the binding data. These tests were done by applying 1 ms or 100 ms pulses of 10 mM glutamate to patches excised from HEK293 cells expressing either wildtype or mutant receptors. As shown in figure 2, several response parameters showed statistically significant changes in mutant receptors. For instance, desensitization was nearly 20% faster (time constants: 2.03 ± 0.06 ms vs 2.48 ± 0.11 ms for wildtype, $n=30/22$, $p<0.001$), and a similar acceleration was apparent in the deactivation time constant (1.08 ± 0.06 ms vs 1.32 ± 0.10 ms, $n=30/21$, $p<0.05$). Even larger changes were seen in the EC_{50} for the peak current, which was increased 1.8 times in mutant receptors (1.8 ± 0.1 mM vs 1.0 ± 0.1 mM, $p<0.001$), and in the ratio between steady-state and peak current which was increased 2.6 times (1.8 ± 0.2 % vs 0.7 ± 0.1 %, $p<0.001$). Lastly, current density was reduced by nearly half in mutant receptors. Figure 2C shows that physiological responses were blocked by guanine nucleotides with inhibition profiles similar to those in binding tests. Responses to 1 ms pulses of glutamate were inhibited by GDP with IC_{50} values of 0.40 mM in wildtype GluR1i and 2.4 mM in GluR1i-K445A. These values are slightly higher than in binding tests, perhaps due to differences in assay temperature. Also, the extent of inhibition at any given nucleotide concentration may have been underestimated because of partial dissociation of GDP while glutamate was present. This is suggested by the observation that the time-to-peak was prolonged in the presence of GDP and that inhibition became weaker if longer glutamate pulses were applied (not shown). Most importantly, however, the apparent affinity for GDP was reduced in mutant receptors to a similar degree as in binding assays.

To characterize further the nature of nucleotide binding, several analogs of GDP were examined (table 3). Surprisingly, removal of the two free hydroxy-groups of ribose did not appear to reduce affinity. This suggests that the receptor does not interact with the ribose moiety of the nucleotide, or only with its ring atoms. Our data further indicate that the nucleotide binding site can accommodate bulky substituents in lieu of the third phosphate, as indicated by the only modest loss in affinity for GDP-mannose and GDP-fucose. The latter finding suggests that the phosphates and attached sugars face in a direction in which there is little steric hindrance, and it is further of interest because GDP-fucose is present within the Golgi cisternae and thus could provide protection against activation by luminal glutamate. In a further set of experiments we attempted to find additional amino acids involved in nucleotide binding. To this end we introduced into R1i-K445A the additional mutations Y446F, N457A, P474A, and T478A in the upper lip of the clamshell and T645A, E653A, T681A and Y698A in the lower lip. Most of these amino acids surround those known to be involved in agonist binding. However, no major change in the affinity for GDP was found following any of these mutations (generally less than two-fold compared to the affinity in R1i-K445A; data not shown). Other mutations, like G447A, L475A and L646A, produced complete loss in radioagonist binding and hence GDP affinity could not be assessed.

PPNDS and suramin are two of several poly-anionic aromatic sulfonates that have recently been shown to allosterically modulate agonist binding to AMPA receptors, with suramin causing an inhibition and PPNDS an increase in agonist binding (Suzuki et al., 2004). Since PPNDS is thought to competitively antagonize nucleotide binding in other proteins (Lambrech et al., 2000), we hypothesized that its docking site on the AMPA receptor would overlap with that for guanine nucleotides and involve some of the same residues on the receptor, particularly those that bind phosphate. Figure 3 shows that this is not the case. The affinity of R1i-K445A receptors for PPNDS was not significantly different from that in wildtype receptors ($4.8 \pm 0.7 \mu\text{M}$ vs $3.9 \pm 0.8 \mu\text{M}$, $p=0.38$, $n=2$); similar results were obtained for suramin (not shown). Secondly, PPNDS at concentrations up to 1 mM, which is 250-fold above its EC_{50} for the AMPA receptor, failed to produce a significant right-shift in the inhibition curve for GDP (figure 3B). These data suggest that - unlike in other receptor classes - PPNDS and nucleotides bind to distinct domains of the AMPA receptor.

DISCUSSION

Inhibition by nucleotides is a universal feature of ionotropic glutamate receptors, but its physiological function remains to be established. Addressing this question may require different experimental approaches depending on the hypothesis to be tested. Thus, receptor modulation by synaptically released guanine nucleotides could be most compellingly demonstrated with compounds that selectively block nucleotide binding to the receptor. Unfortunately, no such agents are presently known, and neither PPNDS nor ribose-phosphate, both of which were examined in this study, proved to be lead compounds suitable for the development of such inhibitors. Testing the alternative hypothesis that nucleotide inhibition evolved to attenuate endo-excitotoxicity may depend on creating receptor variants in which nucleotide binding is eliminated through mutagenesis, which in turn requires to identify amino acids that are selectively involved in nucleotide binding and do not participate in agonist binding or other receptor operations. Our data, combined with those from other studies, indicate that K445 is of particular relevance in this regard and that its role is conserved across species and receptor variants. Specifically, we have found that changing K445 to alanine in rat GluR1 receptors produces a four to six fold reduction in the affinity for GDP and GTP without any change in the binding affinity for the agonists AMPA and fluorowillardiine. These findings match closely those previously reported for the chick kainate binding protein by Paas et al. (1996). In physiological recordings, however, several other receptor properties were found to be altered by the mutation. Changes in desensitization and deactivation were modest, but we observed a significant decrease in the apparent physiological affinity for glutamate, like in the study of Li et al. (1995). Thus, the EC_{50} of glutamate for the peak current was reduced nearly two-times. The latter is not necessarily at variance with the binding data since the dissociation constant K_D is measured at equilibrium while the 'physiological affinity' was determined for the peak transient. We also observed a reduction in the peak current density and in the binding B_{max} by about half, the reason for which is unclear because Western blots did not reveal a decrease in total or surface expression of GluR1 (data not shown).

Nonetheless, the largest changes were by any measure those in the affinity for the guanine nucleotides. This change in affinity was highly dependent on the number of phosphates. Thus, the affinity for GMP was reduced only 1.4 times while that for GDP dropped 4.4 times and that for GTP 5.8 times. This suggests that the second phosphate group benefits most from the presence of the lysine in the native receptor. The most straightforward explanation for this may be that the second nucleotide phosphate is positioned close to the positively charged side chain amino group of lysine 445 and is contacted by the latter through ionic interactions and hydrogen bonds. The electrostatic field generated by K445 would presumably reach far enough to interact also with the negative charges of the first and third phosphate and thereby provide an additional gain in binding energy, which would explain the slightly higher affinity for GMP of wildtype

versus mutant receptors, as well as the larger difference between wildtype and mutant receptors in the affinity for GTP compared to GDP. If this interpretation is correct then guanine nucleotides presumably bind in such a way that the guanine base faces towards the back of the cleft of the S1–S2 module (Armstrong and Gouaux, 2000) - taking the position normally occupied by agonists and competitive antagonists like DNQX - while the phosphates point outward toward the open side of the cleft. This could readily explain why even bulkier compounds like GDP-mannose and GDP-fucose could be accommodated with only minor loss in affinity. It should be noted, however, that the binding energy provided by this postulated K445-GDP interaction would largely be used to compensate for an otherwise unfavorable interaction between the receptor and the nucleotide, as indicated by the progressive loss in affinity in K445A receptors as the number of phosphates increased. Such an unfavorable interaction could result for instance from electrostatic repulsion arising from negatively charged residues near the lips of the cleft, such as for instance from D446 adjacent to K445, or D651 across the cleft. The lower affinity of GTP for wildtype R1i is supportive of such an interaction.

A slightly different docking mode for GMP has recently been proposed by Mendieta et al. (2005) based on molecular dynamics simulations. In their model, the first phosphate group of GMP faces rather towards the side of the cleft, taking roughly the position normally occupied by the α -carboxy group of glutamate (Armstrong and Gouaux, 2000), and interacting with Arg 481 (in GluR1) and with the positive charge of the F-helix dipole. Because their modeling was carried out only for GMP, it remains to be shown that GDP and GTP and the even bulkier GDP-hexoses would be readily accommodated in the proposed configuration, in particular if guanine nucleotides promote cleft closure as suggested in their study. It should be noted that the second and third phosphates of GTP in their proposed docking mode would be expected to protrude from the side of the cleft and hence would be somewhat removed from the amino group of K445 which is positioned rather in the center of the upper rim of the cleft. Further modeling will be needed to determine if bendings and rotations in either the pyrophosphate chain of GDP and/or in the side chain of lysine would allow the second phosphate group and the amino group of K445 to come into proximity without undue strain and energy cost. Some other disparities would have to be reconciled, such as that the 3'-hydroxy group of ribose forms a hydrogen bond with the receptor in their model but does not contribute to nucleotide binding according to our binding assays. Of course, the possibility may also have to be considered that K445 stabilizes nucleotide binding in an indirect manner. In kainate receptors, the lysine that is equivalent to K445 of GluR1 has recently been shown to stabilize cleft closure upon agonist binding by forming a salt bridge with an aspartate on the opposite side of the cleft (Weston et al., 2006). However, the authors detected no such stabilizing influence in S1–S2 constructs from AMPA receptor subunits which have a serine (GluR2) or alanine (GluR1) in lieu of the aspartate. Also, the serine in GluR2 was too distant for any direct bonding with the lysine amino group. Naturally, cleft closure may be different in intact receptors or K445 may stabilize cleft closure through longer range electrostatic interactions. The loss of such stabilization in mutant receptors might in this case explain the acceleration in desensitization and deactivation seen in this study. The question would then remain, however, why such a stabilization would confer a disproportionally large gain in affinity to GDP and GTP.

Paas et al. (1996) in their study also noted that K445 is contained in a sequence which is similar to the GXGXXG motif in other classes of nucleotide binding proteins, including protein kinases and G-proteins. The presence of this motif in glutamate receptors might thus be indicative of homologous function, if not shared evolutionary origin. Some considerations cast doubt on this notion, however. In essentially all nucleotide binding proteins, the GXGXXG motif forms a turn between either two beta-strands, or between a beta sequence and an alpha-helix (Bellamacina, 1996; Noel et al., 1993; Hanks and Hunter, 1995), but in AMPA receptors it is not flanked by either of these secondary structures. Also, in other nucleotide binding proteins

the glycines are the critical elements while the residues between them vary and do not seem to have a conserved function. Specifically, the second and/or third glycines appear to be important for binding phosphate because they permit formation of a hydrogen bond between the latter and the backbone nitrogen, which may be stabilized further by a helix dipole if the glycine is at the beginning of an α -helix. In glutamate receptors, on the other hand, it is a side-chain amino group which appears to assist in nucleotide binding and there is presently no evidence for a role of the glycines. For instance, mutating the second glycine in the motif to alanine (G444A) had no effect on GDP affinity (data not shown), and GluR1 contains a serine at the position normally occupied by the first glycine (S442). Whether the third glycine is involved in GDP binding could not be examined because changing it to alanine (G447A) abolished agonist binding (data not shown), as predicted by Paas et al (1996) based on Ramachandran plot data. There are other reasons to question a deeper homology in nucleotide binding. For instance, evidence suggests that PPNDs is a competitive antagonist of nucleotide binding in purinergic receptors but our data indicate that its binding to AMPA receptors does not overlap with that for nucleotides.

In conclusion, our studies have confirmed that the highly conserved lysine within the (G) XGXXG sequence has a special role in stabilizing the binding of guanine nucleotides, probably by binding the second phosphate. Thus expressing AMPA receptors mutated in this lysine in neurons potentially provides an avenue to elucidate the functional role of nucleotide binding. However, the interpretation of such studies might be confounded by the fact that the apparent physiological affinity for glutamate is also reduced, both under equilibrium conditions (Li et al., 1996) and during current transients similar to those occurring in synaptic transmission (this study). It may thus be important to identify further amino acids with a higher degree of selectivity for the binding of nucleotides. Locating such amino acids evidently would be facilitated if the precise docking mode were unambiguously identified, for instance through crystallography.

EXPERIMENTAL PROCEDURE

Plasmids and mutagenesis

Dr. K. Partin (Fort Collins, CO) generously provided the cDNA for GluR1 flip in the mammalian expression vector pRK5. Mutations were created using the Quick Change® II Site directed mutagenesis Kit from Stratagene (Cedar Creek, TX) following manufacturer's instructions. Lysine 445 of GluR1i (numbering of mature protein) was mutated to alanine using the primers 5'-GATTGTCAGCGACGGCGCATATGGAGCCCGG-3' and 5'-CCGGGCTCCATATGCGCCGTCGC TGACAATC-3'. The primers were obtained from Sigma/Genosys (Woodlands, TX) and designed to include a new Nde I restriction enzyme site to confirm the mutation; the nucleotide changes made to introduce the Nde I site did not alter the amino acid sequence. Plasmids were expanded in E coli and extracted using the Plasmid Maxi Kit from Qiagen (Valencia, CA).

Transfection of HEK293 cells

HEK293 cells grown in a 10 cm culture dish to 70–90% confluency were transfected with 15 μ g plasmid DNA plus 25 μ l Lipofectamine 2000 in Opti-MEM (Invitrogen) serum free medium. The transfection mixture was replaced with normal culture medium containing 100 μ M DNQX after 16–18 hours. Cells were harvested 40–50 hours after start of transfection.

Binding Assays

HEK293 cells were harvested in their culture medium at 0°C, spun down (5 min at 500g) and resuspended in Harvest Buffer (HB; 150 mM NaCl, 10 mM Tris, 0.1 mM EGTA, pH 7.4) plus 0.1% saponin to permeabilize membranes and to remove endogenous glutamate. The cells were

washed once and left in HB for at least one night. Before the binding experiment, the cells were washed again and resuspended in Hepes-buffered saline (HBS; 150 mM NaCl, 20 mM HEPES pH 7.4). In all, permeabilized cells were washed at least three times. Rat brain membranes were prepared using a conventional protocol involving homogenization in sucrose, differential centrifugation, osmotic lysis, and repeated washing (Kessler and Arai, 2005). To measure binding, frozen aliquots were thawed, tip-sonicated, extensively washed, and suspended in HBS. To measure binding, membrane aliquots (5–50 µg protein) were mixed with radioligand and appropriate additions (in HBS) in a final volume of 50 µl and incubated at 0°C for 20–60 min. The incubations were terminated by addition of 5 ml ice-cold buffered saline plus 50 mM potassium thiocyanate (wash buffer) and immediate filtration through Whatman GF/A or GF/C filters. Filters were quickly rinsed with an additional 15 ml of wash buffer and placed in scintillation fluid to determine radioactivity content. Non-specific binding was determined by inclusion of 10 mM glutamate and subtracted from total binding. Protein was measured according to the method of Bradford (1976) using bovine serum albumin as standard. Binding curves were created by fitting the data with nonlinear regression using the Prism program from GraphPad (Inc. San Diego, CA.)

Physiology

Currents mediated by homomeric GluR1 flip receptors were measured in HEK293 cells transiently transfected as described above. Cells were submitted to experimentation 72 to 96 hours after transfection. Patch pipettes had a resistance of 2–5 MΩ and were filled with a solution of (in mM) CsF 130, EGTA 10, ATP-Mg²⁺ 2, spermine 0.1, and HEPES 10 (pH 7.3). The extracellular solution contained: NaCl 140, KCl 3, CaCl₂ 2, MgCl₂ 1, glucose 5, and HEPES 10 (pH 7.3). Patches were excised from the cells and positioned in front of double pipette delivering a constant flow of background medium (without or with nucleotide) in one flow line and medium containing L-glutamate in the second flow line. The patch was initially placed in the background flow line. A piezo device then moved the double pipette in a fraction of a millisecond such that patch became exposed to the glutamate flow line (Arai et al., 1996). The holding potential was -70 mV. Data were acquired with a patch amplifier (AxpPatch-1D) at a filter frequency of 5 kHz and digitized at 10 kHz with PClamp/Digidata 1322A (Axon Instruments).

Materials

[³H]Fluorowillardiine (36 Ci/mmol) was obtained from Tocris (Ballwin MO), S-[³H]AMPA (40.8 Ci/mmol) from Perkin-Elmer/NEN (Boston, MA). Guanine nucleotides and common laboratory reagents were from Sigma-Aldrich (St. Louis, MO), PPNDs was obtained from Tocris (Ballwin, MO).

Acknowledgements

This research was supported by grant NS41020 from the National Institutes of Health. The authors wish to thank Dr. K. Partin (Fort Collins, CO) for providing the plasmid for GluR1.

References

- Arai A, Kessler M, Ambros-Ingerson J, Quan A, Yigiter E, Rogers G, Lynch G. Effects of a centrally active benzoylpyrrolidine drug on AMPA receptor kinetics. *Neuroscience* 1996;75:573–585. [PubMed: 8931020]
- Armstrong N, Gouaux E. Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. *Neuron* 2000;28:165–181. [PubMed: 11086992]

- Baron BM, Dudley MW, McCarty DR, Miller FP, Reynolds IJ, Schmidt CJ. Guanine nucleotides are competitive inhibitors of N-methyl-D-aspartate at its receptor site both in vitro and in vivo. *J Pharmacol Exp Ther* 1989;250:162–169. [PubMed: 2545857]
- Bellamacina CR. The nicotinamide dinucleotide binding motif: a comparison of nucleotide binding proteins. *FASEB J* 1996;10:1257–1269. [PubMed: 8836039]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
- Dev KK, Roberts PJ, Henley JM. Characterisation of the interaction between guanyl nucleotides and AMPA receptors in rat brain. *Neuropharmacology* 1996;35:1583–1593. [PubMed: 9025106]
- Donevan SD, Rogawski MA. GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron* 1993;10:51–59. [PubMed: 7678966]
- Gorodinsky A, Paas Y, Teichberg VI. A ligand binding study of the interactions of guanine nucleotides with non-NMDA receptors. *Neurochem Int* 1993;23:285–291. [PubMed: 7693111]
- Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* 1995;9:576–596. [PubMed: 7768349]
- Kessler M, Rogers G, Arai A. The norbornenyl moiety of cyclothiazide determines the preference for flip-flop variants of AMPA receptor subunits. *Neurosci Lett* 2000;287:161–165. [PubMed: 10854736]
- Kessler M, Arai AC. Use of [³H]fluorowillardiine to study properties of AMPA receptor allosteric modulators. *Brain Res* 2005;1076:25–41. [PubMed: 16256076]
- Lambrecht G, Rettinger J, Baumert HG, Czeche S, Damer S, Ganso M, Hildebrandt C, Niebel B, Spatz-Kumbel G, Schmalzing G, Mutschler E. The novel pyridoxal-5'-phosphate derivative PPNDs potently antagonizes activation of P2X1 receptors. *Eur J Pharmacol* 2000;387:R19–21.
- Li F, Owens N, Verdoorn TA. Functional effects of mutations in the putative agonist binding region of recombinant alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Mol Pharmacol* 1995;47:148–154. [PubMed: 7838123]
- Mendieta J, Gago F, Ramirez G. Binding of 5'-GMP to the GluR2 AMPA receptor: insight from targeted molecular dynamics simulations. *Biochemistry* 2005;44:14470–14476. [PubMed: 16262247]
- Monahan JB, Hood WF, Michel J, Compton RP. Effects of guanine nucleotides on N-methyl-D-aspartate receptor-ligand interactions. *Mol Pharmacol* 1988;34:111–116. [PubMed: 2842650]
- Morciano M, Ortinau S, Zimmermann H. Guanine nucleotides inhibit NMDA and kainate-induced neurotoxicity in cultured rat hippocampal and neocortical neurons. *Neurochem Internat* 2004;45:95–101.
- Noel JP, Hamm HE, Sigler PB. The 2.2 Å crystal structure of transducin-α complexed with GTP γS. *Nature* 1993;366:654–663. [PubMed: 8259210]
- Ortinau S, Laube B, Zimmermann H. ATP inhibits NMDA receptors after heterologous expression and in cultured hippocampal neurons and attenuates NMDA-mediated neurotoxicity. *J Neurosci* 2003;23:4996–5003. [PubMed: 12832522]
- Paas Y, Devillers-Thiery A, Changeux JP, Medevielle F, Teichberg VI. Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. *EMBO J* 1996;15:1548–1556. [PubMed: 8612578]
- Pinton P, Pozzan T, Rizzuto R. The Golgi apparatus is an inositol 1,4,5-triphosphate-sensitive Ca²⁺ store, with functional properties distinct from those of the endoplasmic reticulum. *EMBO J* 1998;17:5298–5308. [PubMed: 9736609]
- Santos TG, Souza DO, Tasca CI. GTP uptake into rat brain synaptic vesicles. *Brain Res* 2006;1070:71–76. [PubMed: 16405924]
- Suzuki E, Kessler M, Montgomery K, Arai AC. Divergent effects of the purinoceptor antagonists suramin and pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDs) on AMPA receptors. *Mol Pharmacol* 2004;66:1738–1747. [PubMed: 15448189]
- Weston MC, Gertler C, Mayer ML, Rosenmund C. Interdomain interactions in AMPA and kainate receptors regulate affinity for glutamate. *J Neurosci* 2006;26:7650–7658. [PubMed: 16855092]
- Willard JM, Ziegra CJ, Oswald RE. The interaction of a kainate receptor from goldfish brain with a pertussis toxin-sensitive GTP-binding protein. *J Biol Chem* 1991;266:10196–10200. [PubMed: 1674742]

Willard JM, Oswald RE. Interaction of the frog brain kainate receptor expressed in Chinese hamster ovary cells with a GTP-binding protein. *J Biol Chem* 1992;267:19112–19116. [PubMed: 1326545]

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA	N-methyl-D-aspartate
FW	fluorowillardiine
PPNDS	pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate)
HBS	HEPES-buffered saline

Figure 1. Agonist binding and guanine nucleotide inhibition in wildtype GluR1 flip versus GluR1i-K445A

A. Binding of [3 H]FW was measured at 0°C at radioligand concentrations of 1–100 nM. Non-specific binding was determined by inclusion of 10 mM glutamate and subtracted from total binding. Specific binding was transformed into the Scatchard format; linear regression provided the K_D values shown as insets. Representative experiment; for summary data see table 1. **B.** Inhibition of [3 H]FW binding (5 nM, 0°C) to wildtype (wt) R1i receptors by guanine nucleotides and guanosine. Binding was expressed as percent of that in the absence of nucleotide and averaged across experiments. The averaged data (mean and sem) were then fitted through non-linear regression with a sigmoidal inhibition curve ($n_{Hill} = 1$; bottom asymptote fixed at 0). **C.** Comparison of nucleotide inhibition in wildtype R1i and R1i-K445A (mut). The data for the wildtype receptor are the same as in B. The inhibition curves were fitted to the averaged data. The insets show the averaged inhibition constants K_i as taken from table 1.

Figure 2. Physiological properties of GluR1-K445A

Membrane patches were excised from HEK293 cells transfected with GluR1-flip wildtype (wt) or GluR1-K445A (K445A). Typically, 5–10 consecutive traces were averaged. The holding potential was -70 mV. **A.** Concentration response relations for glutamate. Pulses of various concentrations of glutamate were applied for 100 ms, and peak amplitude at each concentration were normalized to that at 10 mM glutamate. The data points represent values obtained in individual experiments ($n=5$ for wildtype, $n=6$ for K445A). The EC_{50} value was calculated by fitting the data points with a sigmoidal function. The insets show representative traces (0.3–10 mM). The horizontal bar indicates application of glutamate. **B.** Changes in kinetic parameters. Desensitization and deactivation kinetics were examined in currents induced by 100 ms and 1 ms pulses of glutamate (10 mM), respectively. The time constants were determined by fitting the decay phase of the response with a single exponential function. % SS/Peak denotes the percentage of steady-state current relative to the peak current. Only responses with a peak current higher than 100 pA were analyzed. The bars show the mean and s.e.m. of 30 (K445A) and 21–22 (wild type) experiments; current density for R1i-K445A was determined in 40 experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **C.** Concentration-response relations for GDP. The nucleotide was included at the indicated concentrations in both the background flow line and in the glutamate pulse (10 mM). Left: representative traces; horizontal bars indicate glutamate application. Right: Summary data for the effect of GDP on peak amplitude. The data points represent the mean and sem from 7 (K445A) and 8 (wildtype) experiments. The data were fitted with a sigmoidal function; IC_{50} values are shown in the insets.

Figure 3. Lack of interactions between PPNDS and guanine nucleotides

Left. Enhancement of [³H]AMPA binding to wildtype GluR1i and GluR1i-K445A by PPNDS. Membranes were incubated for 30–60 min at 0°C with 20 nM [³H]AMPA (without potassium thiocyanate) and the PPNDS concentrations indicated on the abscissa. For each experiment, binding was normalized to that in the absence of PPNDS (1.25 pmol/mg protein for wt and 0.52 pmol/mg for mutant receptors); non-specific binding was 10–20% of total binding. Averaged data (mean and sem, n=2) were then fitted with a four-point logistic equation. EC₅₀ values for PPNDS are shown as insets; Hill coefficients were 1.4 for both receptors.

Right. Inhibition of [³H]FW binding to brain AMPA receptors by GDP in the absence and presence of 100 μM PPNDS. Membranes were incubated for 60 min at 0°C with 20 nM [³H]FW and the indicated GDP concentrations. PPNDS was added to the membranes together with GDP and [³H]FW. The data were normalized, averaged (mean and sem, n=3), and fitted with a logistic equation (n_{Hill} = 1). The IC₅₀ values are shown as insets. The ~1.5-fold shift in the IC₅₀ for GDP was also seen in tests with 1 mM PPNDS (not shown) and is due to the increase in affinity for [³H]FW caused by PPNDS. If PPNDS and GDP interactions were competitive then the IC₅₀ for GDP should have shifted about 25-fold at 100 μM PPNDS and about 250-fold at 1 mM PPNDS. Absence of competition was similarly noted in tests with the bulkier nucleotides GTP and GDP-mannose (not shown).

Table 1
Binding constants for agonists and guanine nucleotides

	GluR1i	GluR1i-K445A	N	ratio	
K _D for [³ H]fluorowillardiine (nM)	7.1 ± 0.6	5.9 ± 0.6	4	0.83	NS
K _i for guanosine (μM)	1165 ± 100	1012 ± 35	2	0.87	NS
K _i for GMP (μM)	229 ± 10	309 ± 19	3	1.35	p = 0.02
K _i for GDP (μM)	215 ± 4	937 ± 208	4	4.4	p = 0.04
K _i for GTP (μM)	311 ± 24	1797 ± 197	4	5.8	p=0.005

Summary data for the experiments of figure 1. Dissociation constants K_D for FW were determined by fitting a hyperbolic function to the binding at 1 – 100 nM [³H]FW; the values shown in the table are means and sem from N paired experiments. The inhibition constants K_i for nucleotides and guanosine were determined by fitting inhibition data as shown in figure 1B and C with a sigmoidal function (nHill = 1; bottom asymptote fixed at 0) and multiplying the IC₅₀ value obtained from this curve fit with a Cheng-Prusoff factor to correct for the competitive displacement of the nucleotide by the radioagonist. The correction factors (1 / (1 + [FW]/[K_D of FW])) were 0.59 for wildtype and 0.54 for mutant receptors. K_i values were determined separately for each inhibition experiment and then averaged to give the mean and sem values shown in the table. Values for p were determined using Student's t-test, in some cases (GDP, GTP) with Welch's correction for unequal variances. NS: not significant.

Table 2
Binding constants for GDP at other AMPA receptor subunits and brain AMPA receptors

	K_i	N
GluR1i	215 ± 4	4
GluR1o	192 ± 4	2
GluR2o	82 ± 1	3
GluR2i	98 ± 8	2
GluR3i	103 ± 7	3
GluR4i	212 ± 18	3
rat brain membranes (IC_{50})	228 ± 18	4

Inhibition constants for GDP were determined as described in figure 1 and table 1. For GluR1-4, the K_i constants were determined by multiplying the IC_{50} values with a Cheng-Prusoff correction factor. No correction was applied for binding to brain membranes because brain receptors exhibit two or more affinity components (22 nM and 964 nM in two-site fits; Kessler and Arai, 2005). Subunits other than GluR1i are stably expressed in HEK293 cells (Kessler et al., 2000).

Table 3
Inhibition of fluorowillardiine binding to rat brain membranes by other nucleotides

	IC ₅₀ (μM)	N
GDP	228 ± 18	4
2'-deoxy-GDP	209 ± 15	2
2',3'-dideoxy-GTP	231 ± 9	3
GDP-mannose	389 ± 5	3
GDP-fucose	394 ± 13	4
Cyclic GMP	612 ± 14	2
IMP	2758 ± 195	3

Inhibition constants were determined in rat brain membranes by measuring binding of [³H]fluorowillardiine (20 nM) at 0°C in the presence of 3–5 concentrations of the nucleotide and fitting a sigmoidal inhibition curve (with $n_{\text{Hill}} = 1$) to the data points. No Cheng-Prusoff correction was applied because brain receptors exhibit two or more affinity components.

In additional experiments (not shown), no detectable inhibition was observed with 1 or 2 mM ATP, UDP-glucuronic acid or UDP-N-acetyl-galactosamine. Inhibition by GDP was not significantly influenced by inclusion of 100 μM Ca²⁺, 2 mM Mg²⁺, 300 μM Zn²⁺, 3.2 mM CX546 (positive AMPA receptor modulator; Kessler and Arai, 2005), or 200 μM GYKI 52466 (negative modulator; Donevan and Rogawski, 1993). Also, 5–10 mM ribose-5'-phosphate did not significantly inhibit [³H]fluorowillardiine binding (<3%) and it did not antagonize the inhibition by GDP.