

A Novel Allosteric Potentiator of AMPA Receptors: 4-[2-(Phenylsulfonylamino)ethylthio]-2,6-Difluoro-Phenoxyacetamide

Masayuki Sekiguchi,¹ Mark W. Fleck,² Mark L. Mayer,² Jiro Takeo,³ Yoshiyuki Chiba,³ Shinya Yamashita,³ and Keiji Wada¹

¹Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187, Japan, ²Laboratory of Cellular and Molecular Neurophysiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4495, and ³Central Research Laboratory, Nippon Suisan Kaisha Limited, Hachioji, Tokyo 192, Japan

We report that a novel sulfonylamino compound, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA), selectively potentiates glutamate receptors of the AMPA subtype. PEPA (1–200 μM) dose dependently potentiated glutamate-evoked currents in *Xenopus* oocytes expressing AMPA (GluRA–GluRD), but not kainate (GluR6 and GluR6+KA2) or NMDA ($\zeta 1 + \epsilon 1$ – $\epsilon 4$), receptor subunits. PEPA was effective at micromolar concentrations and, in contrast to the action of cyclothiazide, preferentially modulated AMPA receptor flop isoforms. At 200 μM , PEPA potentiated glutamate responses by 50-fold in oocytes expressing GluRC_{flop} ($\text{EC}_{50} \sim 50 \mu\text{M}$) versus only threefold for GluRC_{flip}; a similar preference for flop isoforms was observed for other AMPA receptor subunits. Dose–response analysis for GluRC_{flop} revealed that 100 μM PEPA produced a sevenfold increase in AMPA recep-

tor affinity for glutamate. PEPA produced considerably weaker potentiation of kainate-evoked than glutamate-evoked currents, suggesting modulation of the process of receptor desensitization. In human embryonic kidney 293 cells transfected with AMPA receptor subunits, PEPA either abolished or markedly slowed the rate of onset of desensitization and potentiated steady-state equilibrium currents evoked by glutamate with subunit (GluRC \geq GluRD > GluRA) and splice-variant (flop > flip) selectivity similar to that observed in oocytes. Our results show that PEPA is a novel, flop-preferring allosteric modulator of AMPA receptor desensitization at least 100 times more potent than aniracetam.

Key words: glutamate receptors; AMPA; desensitization; alternative splicing; flip and flop; allosteric modulation

Allosteric modulation of the three subtypes of ionotropic glutamate receptors—AMPA, kainate, and NMDA receptors—is produced by a diverse spectrum of agents, including lectins, a variety of drugs, polyamines, and divalent cations. The unusually strong modulation of AMPA receptors by the benzothiadiazine and pyrrolidinone compounds cyclothiazide, aniracetam, and their derivatives (Ito et al., 1990; Isaacson and Nicoll, 1991; Tang et al., 1991; Vyklicky et al., 1991; Hestrin, 1992; Yamada and Rothman, 1992; Bertolino et al., 1993; Patneau et al., 1993; Yamada and Tang, 1993; Arai et al., 1994; Staubli et al., 1994a,b) is especially interesting, because these drugs potentiate excitatory synaptic transmission and have the potential for therapeutic use as nootropic agents.

AMPA receptors are hetero-oligomeric complexes, most likely pentamers (Wenthold et al., 1992; Ferrer-Montiel and Montal, 1996), generated by the assembly of various combinations of four subunits named GluRA, GluRB, GluRC, and GluRD or GluR1, GluR2, GluR3, and GluR4, respectively (Hollmann et al., 1989;

Boulter et al., 1990; Keinänen et al., 1990). Each subunit exists in flip and flop isoforms generated by alternative splicing (Sommer et al., 1990), and their expression is regulated both regionally and developmentally (Boulter et al., 1990; Keinänen et al., 1990; Sommer et al., 1990; Monyer et al., 1991). Because the assembly of AMPA receptors containing more than one type of subunit appears not to require a fixed stoichiometry, it is possible to generate a diverse array of receptor subtypes that differ in important functional properties. For example, subunit composition can affect the affinity for various agonists and antagonists (Stein et al., 1992). Splice-variant composition affects the kinetics of receptor deactivation, the rate of onset and recovery from desensitization, and modulation by cyclothiazide and aniracetam (Lomeli et al., 1994; Mosbacher et al., 1994; Partin et al., 1994). Assembly of GluRB with other subunits reduces Ca^{2+} permeability (Hollmann et al., 1991; Hume et al., 1991; Sommer et al., 1991), single-channel conductance (Swanson et al., 1997), and rectification resulting from channel block by polyamines (Verdoorn et al., 1991; Bowie and Mayer, 1995).

Although the physiological significance of the cell-specific expression of AMPA receptor subunits and splice variants is not yet fully understood, it is likely that the regulation of receptor composition determines the kinetics and strength of transmission at glutamatergic synapses. As such, drugs that modulate AMPA receptors are both useful pharmacological tools as well as potential therapeutic agents. The modulation of AMPA receptors by aniracetam and cyclothiazide, the most thoroughly examined agents, reveals profound differences in potency, pharmacological selectivity, and mechanism of action. Aniracetam is effective at

Received March 14, 1997; revised May 19, 1997; accepted May 20, 1997.

This investigation was supported in part by research grants from the Ministry of Education, Science, Sports, and Culture, the Ministry of Health and Welfare, the Science and Technology Agency of Japan, and the Japan Foundation for Neuroscience and Mental Health. We thank Drs. S. Heinemann, M. Mishina, and P. Seeburg for cDNAs; Drs. M. Hollmann and R. Wenthold for their critical comments; and Drs. T. Nishikawa and K. Takahashi for comments and amino acid analysis via HPLC.

Correspondence should be addressed to Dr. Masayuki Sekiguchi, Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan.

Copyright © 1997 Society for Neuroscience 0270-6474/97/175760-12\$05.00/0

millimolar concentrations and preferentially modulates flop splice variants (Johansen et al., 1995; Partin et al., 1996), whereas cyclothiazide is effective at micromolar concentrations and preferentially modulates flip splice variants (Partin et al., 1994, 1996).

Here we report that a novel sulfonylamino compound, PEPA, which is structurally distinct from the previously characterized pyrrolidinone or benzothiadiazine compounds typified by aniracetam and cyclothiazide (see Fig. 1), potentiates currents of recombinant AMPA receptors expressed in *Xenopus* oocytes. PEPA is effective at micromolar concentrations, is selective for the AMPA subtype of glutamate receptors, and preferentially modulates flop splice variants. Rapid perfusion experiments in transfected human embryonic kidney 293 (HEK 293) cells show that potentiation by PEPA results primarily from attenuation of desensitization.

MATERIALS AND METHODS

Xenopus oocyte expression system. Glutamate receptor expression in *Xenopus* oocytes was performed as described previously (Sekiguchi et al., 1994). Briefly, cRNA was prepared from linearized cDNA encoding glutamate receptors by *in vitro* transcription. The cDNAs encoding rat GluR1_{flop}, GluR2_{flop}, GluR3_{flop}, GluR6, and KA2 were kindly provided by Dr. Steven Heinemann (The Salk Institute for Biological Studies, San Diego, CA). In Results, these are referred to as GluRA, GluRB, and GluRC to maintain consistent nomenclature with experiments on HEK 293 cells, which were performed with cDNAs from Dr. Peter Seeburg. The flip variants of GluR1, GluR2, and GluR3 were prepared from corresponding flop cDNAs by using site-directed mutagenesis (MutaGene Phagemid kit, Bio-Rad, Hercules, CA) as described previously (Sekiguchi et al., 1994; Matsui et al., 1995). The mutations were confirmed by sequence analysis. Oocyte expression plasmids encoding GluRD_{flop} and GluRD_{flip} were provided by Dr. Seeburg (University of Heidelberg, Heidelberg, Germany). The R/G sites in GluR2_{flip} and GluR3_{flip} (both G versions) are the same as in their original flop clones; the sites in GluRD were R (flip) and G (flop). The cDNAs encoding mouse NMDA receptors were kindly provided by Dr. M. Mishina (University of Tokyo, Tokyo, Japan).

The concentration of the cRNA solution injected into oocytes was 1 mg/ml, and 50 nl of the solution was injected into an oocyte. The ratio of GluRA, GluRC, or GluRD to GluRB was 1:1, unless specified otherwise. Electrophysiological responses were recorded 4–6 d after injection with a two-electrode voltage clamp at a holding potential of -100 mV, unless otherwise specified. Oocytes were perfused with frog Ringer's solution consisting of (in mM): 115 NaCl, 2 KCl, 1.8 CaCl₂, and 10 HEPES, pH 7.2 with NaOH. PEPA was dissolved in dimethylsulfoxide (DMSO), usually at 100 mM, and added to frog Ringer's solution to yield final concentrations of 0.2–200 μ M; the final concentration of DMSO was no more than 0.2%. The pH of the frog Ringer's solution was not changed by adding 200 μ M PEPA. At room temperature no precipitation was observed in the PEPA–DMSO–frog Ringer's solution up to 200 μ M PEPA. Concanavalin A (Sigma, St. Louis, MO) was dissolved in frog Ringer's solution (1 mg/ml), and the oocytes were incubated in this solution for ~ 3 min at room temperature before recording.

Rapid perfusion experiments in HEK 293 cells. Plasmids encoding cDNA clones of rat GluRA, GluRB, GluRC, and GluRD flip (_i) and flop (_o) in CMV expression vectors (gifts from Dr. Peter Seeburg) were prepared by alkaline lysis, followed by 2 \times cesium chloride gradient purification. HEK 293 cells (ATCC CRL 1573) were plated at low density on 35 mm Petri dishes and transfected 24 hr later, using the calcium phosphate precipitation technique of Chen and Okayama (1987). Cells were maintained in MEM with Earle's salts, 2 mM glutamine, and 10% fetal bovine serum. Heteromeric receptors were examined by cotransfection of GluRA, GluRC, or GluRD with GluRB (at a ratio of 1:2); heteromerization was confirmed by analysis of current-voltage plots as described previously (Partin et al., 1994), and data were excluded for cells with significant inward rectification. Whole-cell recordings from isolated HEK 293 cells were obtained 40–72 hr after transfection, using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA), at a holding potential of -60 mV. Extracellular recording solution contained (in mM): 145 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, and 0.01 mg/ml phenol red, pH 7.3, osmolarity 295 mOsm. Borosilicate glass pipettes (WPI 1B150F, World Precision Instruments, Sarasota, FL) had

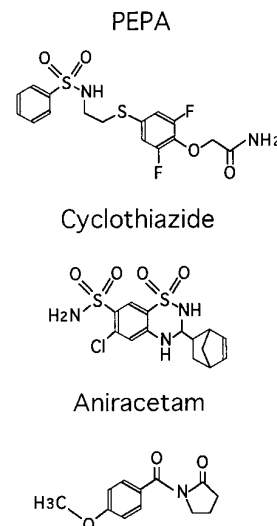


Figure 1. Chemical structures of PEPA, cyclothiazide, and aniracetam.

resistances of 2–5 M Ω when filled with (in mM): 135 CsCl, 10 CsF, 0.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 Cs₄-BAPTA, pH 7.2, osmolarity 295 mOsm. Solutions were applied at a flow rate of 0.67 ml/min controlled by a syringe pump (WPI sp220i) with a stepper motor-based fast perfusion system as described previously (Vyklícky et al., 1991). Responses were filtered at 2–5 kHz (8-pole Bessel filter), digitized as required, and stored on a Macintosh PPC 7600/132 computer by using a 16-bit AD converter (Instrutech ITC-16, Great Neck, NY) under control of the data acquisition and analysis program Synapse (Synergy Research, Silver Spring, MD). The control barrel of the fast perfusion system contained normal extracellular solution; the other barrels contained 3 mM L-glutamate, 100 μ M PEPA, or both. PEPA was dissolved in DMSO at 100 mM before dilution in extracellular solution, and an equivalent final concentration of DMSO (0.1%) was added to all glutamate and control solutions not containing PEPA. The time constant of onset of desensitization (τ_{des}) in the presence and absence of PEPA was fit by a single exponential function for all subunits except GluRA_i, for which responses in the presence of PEPA were better fit by the sum of two exponentials (see Fig. 9A). The onset (τ_{on}) and recovery (τ_{off}) from potentiation in response to rapid application of PEPA (see Fig. 8B, Table 1) were fit with one (τ_{on}) or the sum of two (τ_{off}) exponentials for all subunits.

RESULTS

The action of PEPA (Fig. 1) on glutamate receptors was discovered via electrophysiological screening of compounds containing sulfonylamino groups, using the *Xenopus* oocyte expression system and poly(A⁺) mRNA prepared from rat brain. PEPA was synthesized originally in the process of developing thromboxane A₂ receptor antagonists (Sato et al., 1994, 1995), but the activity of PEPA on A₂ receptors has not yet been tested. PEPA produced small inward current responses in oocytes expressing AMPA receptor flop splice variants but did not evoke current responses in control oocytes prepared from four different frogs and injected with water ($n = 45$). PEPA (200 μ M) injected into oocytes (20 nl/oocyte) also did not elicit current responses ($n = 3$). When the PEPA sample or frog Ringer's solution used in these experiments was analyzed by HPLC (the analysis system could detect concentrations exceeding 10 nM), no contaminating glutamate or aspartate peaks were detected. When it was applied by concentration jump to HEK 293 cells transfected with glutamate receptor subunits, PEPA (100 μ M) failed to activate rapidly desensitizing responses even in cells expressing AMPA receptors at high density. Because subsequent experiments revealed a profound potentiation of glutamate responses by PEPA, we focused our attention on the modulatory action of PEPA and have not yet determined

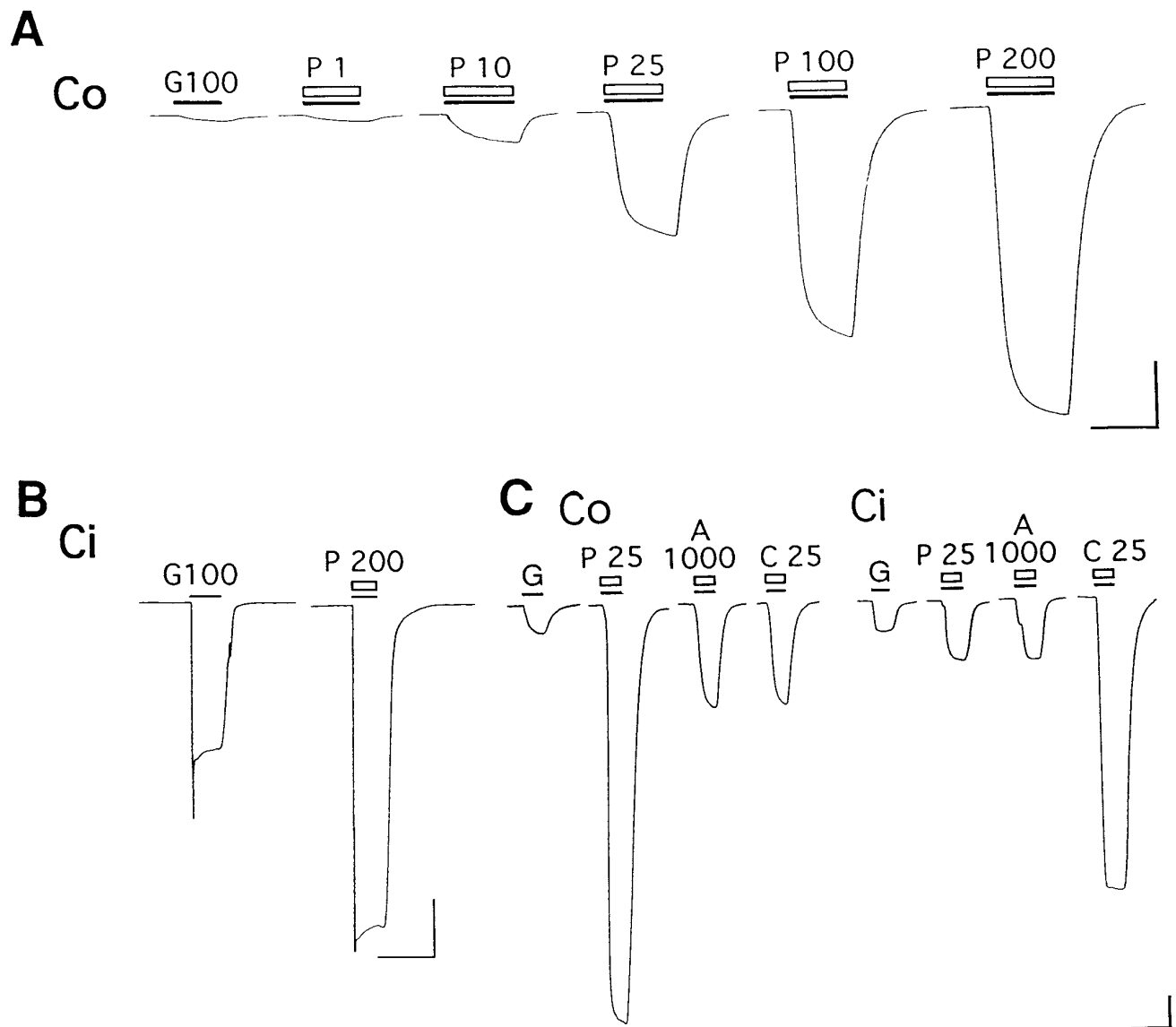


Figure 2. PEPA strongly potentiates glutamate-evoked currents in oocytes expressing GluRC_o. *A, B*, Potentiation by PEPA of glutamate-evoked currents in *Xenopus* oocytes expressing GluRC_o and/or GluRC_i. Glutamate, 100 μM, was applied during the periods indicated by filled bars (G100). PEPA was applied at the indicated concentrations (in μM) during the periods indicated by open bars (P 1–P 200). Holding potential, –70 mV; calibration, 20 sec and 100 nA. *C*, The potentiation of 100 μM glutamate-evoked currents by PEPA [P 25 (μM)], aniracetam [A 1000 (μM)], and cyclothiazide [C 25 (μM)] in oocytes expressing GluRC_o or GluRC_i. Holding potential, –100 mV; calibration: 30 sec and 50 nA for GluRC_o, and 30 sec and 150 nA for GluRC_i.

the mechanism or mechanisms underlying the small amplitude responses to PEPA itself recorded with *Xenopus* oocytes.

PEPA potentiates AMPA receptor responses with opposite splice-variant selectivity to cyclothiazide

Figure 2*A* shows the effects of various concentrations of PEPA on currents evoked by 100 μM glutamate in an oocyte expressing GluRC_o. Glutamate-evoked currents were strongly potentiated by PEPA in a concentration-dependent manner. In contrast, Figure 2*B* shows the much weaker potentiation by 200 μM PEPA of glutamate-evoked currents in an oocyte expressing GluRC_i. The amount of potentiation by PEPA (200 μM) was consistently much greater for GluRC_o (50.2 ± 10.7 -fold, $n = 7$) than for GluRC_i (2.7 ± 0.3 -fold, $n = 10$). Figure 2*C* compares the splice-variant selective action of 25 μM PEPA with the action of 1 mM aniracetam and 25 μM cyclothiazide, two well characterized allosteric modulators of AMPA receptors. PEPA caused much greater

potentiation of glutamate responses in oocytes expressing GluRC_o than either aniracetam or cyclothiazide. In contrast, cyclothiazide caused the greatest potentiation in oocytes expressing GluRC_i. The flop-selective action of PEPA resembles that of aniracetam, but clearly PEPA is considerably more potent; in fact, potentiation by 25 μM PEPA for GluRC_o responses (21.5 ± 3.9 -fold, $n = 7$) was even larger than that produced by 25 μM cyclothiazide for GluRC_i responses (13.4 ± 2.4 -fold, $n = 6$).

In an attempt to define accurately the differences in the potency for modulation of AMPA receptors by PEPA and aniracetam, we performed dose–response analyses in oocytes expressing GluRC_o or GluRC_i. Figure 3 shows potentiation by PEPA and aniracetam expressed as the fold increase in responses to 100 μM glutamate when coapplied with modulator. Both aniracetam and PEPA were flop-preferring and produced much greater potentiation for GluRC_o than for GluRC_i at their limit of solubility in

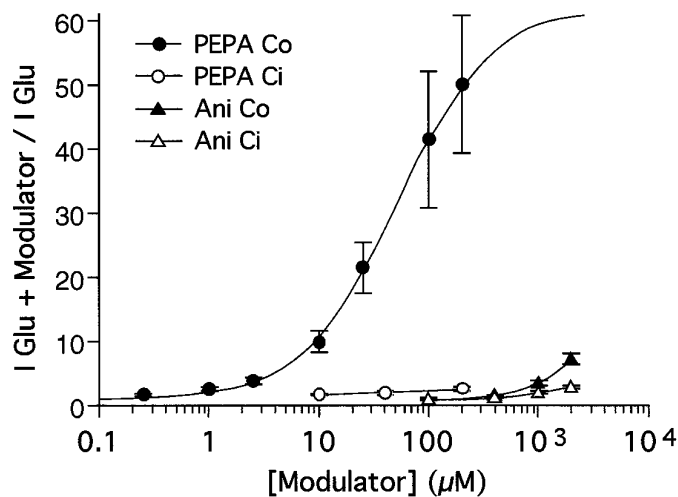


Figure 3. PEPA is much more potent than aniracetam. Shown are dose–response curves for potentiation of glutamate responses by PEPA and aniracetam (*Ani*) in oocytes expressing GluRC_o and GluRC_i. Glutamate (100 μ M) was applied first to oocytes to obtain control responses, and then solutions containing both glutamate and various concentrations of PEPA or aniracetam were applied. *Ordinate*, Fold potentiation is given as the amplitude of the response evoked by coapplication of glutamate plus modulator/amplitude of the response to glutamate alone. In the case of GluRC_o, the dose–response curve for PEPA indicates the best fit to the data according to the logistic function $P = 1 + P_{\max}/[1 + (EC_{50}/[D])^n]$, in which P is the fold potentiation, P_{\max} is the maximal potentiation, EC_{50} is the concentration causing 50% of maximal potentiation, $[D]$ is the concentration of PEPA, and n is the Hill coefficient. The other data were connected simply by lines. The apparent EC_{50} for potentiation of GluRC_o by PEPA was 50.4 μ M (Hill coefficient = 1.03). Values are mean \pm SEM ($n = 6–8$). SEM is smaller than the symbol for data points in which the error bars are not visible.

frog Ringer's solution. The EC_{50} for potentiation of GluRC_o by PEPA was 50 μ M (Hill coefficient = 1.03). Aniracetam did not produce any detectable potentiation at 100 μ M, and at 1–2 mM it produced almost comparable potentiation to only 10 μ M PEPA. Although complete dose–response curves could not be constructed for aniracetam because of its limited solubility and low potency, our data indicate that PEPA was at least 100 times more potent for potentiation of GluRC_o than aniracetam (Fig. 3). Dose–response analysis of PEPA modulation for GluRC_i suffered from similar limitations to those for analysis of the effects of aniracetam, and within the concentration range that it was possible to analyze we were unable to determine whether the maximum potentiation by PEPA for GluRC_i was less than for GluRC_o or whether, in addition, the EC_{50} of PEPA for GluRC_i was greater than the EC_{50} for GluRC_o.

The flop-preferring action of aniracetam has been reported previously for studies comparing GluRA_oB_o with GluRA_iB_i and GluRB_oD_o with GluRB_iD_i expressed in oocytes (Johansen et al., 1995) and for homomeric GluRA_o compared with homomeric GluRA_i expressed in HEK 293 cells (Partin et al., 1996). To determine whether the selectivity of PEPA for GluRC_o versus GluRC_i was maintained in other AMPA receptor subunits, we compared potentiation of responses to glutamate by 200 μ M PEPA for GluRA, GluRC, and GluRD expressed alone and in combination with GluRB. Figure 4 shows that PEPA caused consistently greater enhancement of currents evoked by 100 μ M glutamate in oocytes expressing AMPA receptor flop versus flip isoforms for all subunits examined. However, there were clear differences among subunits and between homomeric receptors

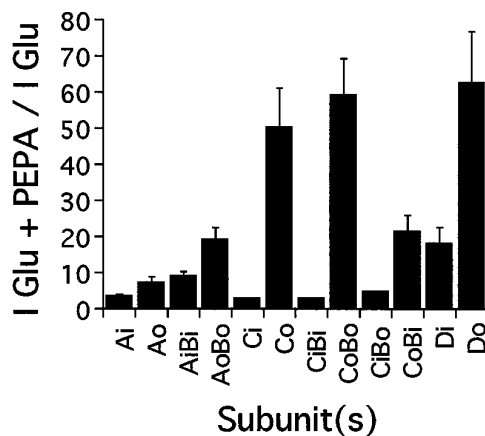


Figure 4. Subunit and splice-variant selectivity of the action of PEPA. Oocytes were injected with the AMPA receptor subunit cRNAs denoted. Glutamate (100 μ M) was applied first to oocytes, and then a solution containing both glutamate (100 μ M) and PEPA (200 μ M) was applied. *Ordinate*, Fold potentiation is given as the amplitude of the response evoked by coplication of glutamate plus PEPA/amplitude of the response to glutamate alone. Values are mean \pm SEM ($n = 5–22$).

versus heteromeric receptors generated by coassembly with GluRB. GluRA_o was the least sensitive of the flop variants, but nonetheless it showed greater potentiation than GluRA_i (Fig. 4). Coassembly with GluRB_o increased potentiation by PEPA for GluRA_o. In heteromeric receptors formed from GluRB and GluRC, the magnitude of enhancement by PEPA was dependent on the splice isoform of GluRC; although marked potentiation was observed for GluRB_oCo_o, only moderate potentiation was observed for GluRB_iCo_o, whereas GluRB_iCi and GluRB_oCi were potentiated only weakly. Because these subunits were injected at a ratio of 1:1, it is possible that the results obtained reflect formation of homomeric GluRC rather than a dominant effect of the GluRC subunit.

PEPA is selective for AMPA subclass ionotropic glutamate receptors

Next, we tested effects of PEPA on other glutamate receptor subtypes. Figure 5A shows traces obtained from two oocytes expressing GluR6, a kainate receptor subunit. The oocyte shown in the top traces was untreated, and that in the bottom traces was treated with concanavalin A to block desensitization, as described in Materials and Methods. For the untreated oocyte, responses to 100 μ M glutamate were separated by 10 min intervals to allow recovery from desensitization; then PEPA (100 μ M) and glutamate were applied simultaneously, and the oocyte was washed with Ringer's solution again for 10 min to confirm recovery. After treatment with concanavalin A, solutions were applied by using the same order but with applications of glutamate separated by 1 min intervals. In contrast to its effects on AMPA receptors, PEPA did not potentiate the glutamate-evoked currents for GluR6 ($n = 10$) either before or after reduction of desensitization by concanavalin A; instead, PEPA caused a modest but reproducible inhibition, which was not observed with the 0.1% DMSO present in 100 μ M PEPA solutions. Similar experiments performed in oocytes expressing the kainate receptor subunits GluR6 plus KA2 ($n = 10$) confirmed that there was also no potentiation by PEPA of these receptors (Fig. 5B). Currents evoked by simultaneous application of glutamate and glycine (10 μ M each) in oocytes expressing the NMDA receptor subunits ζ plus subunits

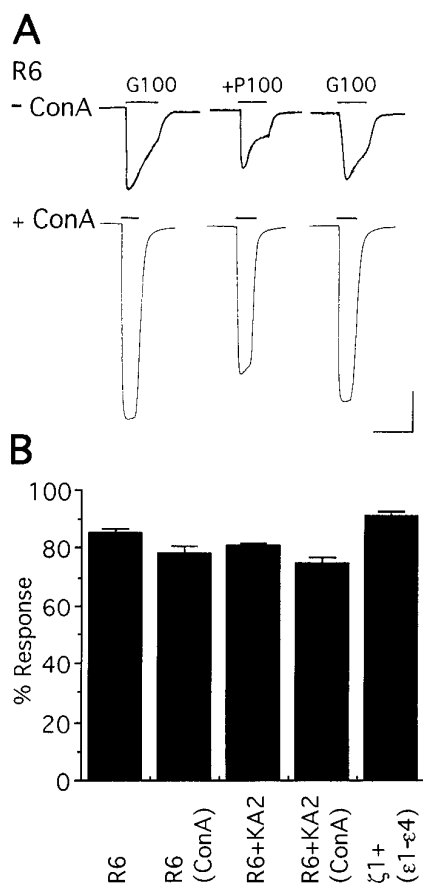


Figure 5. PEPA is selective for AMPA subtype glutamate receptors. *A*, Action of PEPA (100 μ M) on glutamate responses (100 μ M) of oocytes expressing GluR6, which were untreated (- ConA) or pretreated with Concanavalin A (+ ConA) before recording. *Left traces* are control glutamate currents (G100); *middle traces* show responses to coapplication of glutamate + PEPA (+P100); *right traces* show recovery. Calibration: 20 sec for all traces, and 50 nA for - ConA or 100 nA for + ConA. *B*, Similar experiments were performed in oocytes expressing GluR6 + KA2 (1:1) or $\zeta 1 + \epsilon 1 + \epsilon 2 + \epsilon 3 + \epsilon 4$ (4:1:1:1:1). Ordinate, Percentage of response is given as the amplitude of the response evoked by glutamate (100 μ M for kainate receptors and 10 μ M for NMDA receptors) plus PEPA (100 μ M)/amplitude of the response to glutamate alone. Values are mean \pm SEM ($n = 5-15$). In oocytes expressing NMDA receptor subunits, glycine (10 μ M) was coapplied with glutamate.

$\epsilon 1-\epsilon 4$ ($n = 11$) also were not potentiated by PEPA (Fig. 5*B*); a very small response was elicited by glutamate without glycine in these oocytes, which also was not potentiated by PEPA (data not shown).

PEPA increases GluR_C apparent affinity for glutamate

Cyclothiazide has been shown previously to cause a leftward shift in the agonist dose-response relationship for native AMPA receptors in hippocampal neurons and for GluR_A_i expressed in oocytes (Patneau et al., 1993; Yamada and Tang, 1993; Partin et al., 1994); in contrast, it has been reported that aniracetam produces a shift to the right of the glutamate dose-response relationship for GluR_A_o (Tsuzuki et al., 1992). In similar experiments we observed that PEPA (10-100 μ M) causes a leftward shift in the glutamate dose-response relationship in oocytes expressing GluR_C (Fig. 6*A,B*). The apparent EC₅₀ and Hill coefficient values for glutamate were, respectively, $36 \pm 6 \mu$ M and 1.2 ($n = 7$) without PEPA, $10 \pm 1 \mu$ M and 1.7 ($n = 7$) in the presence

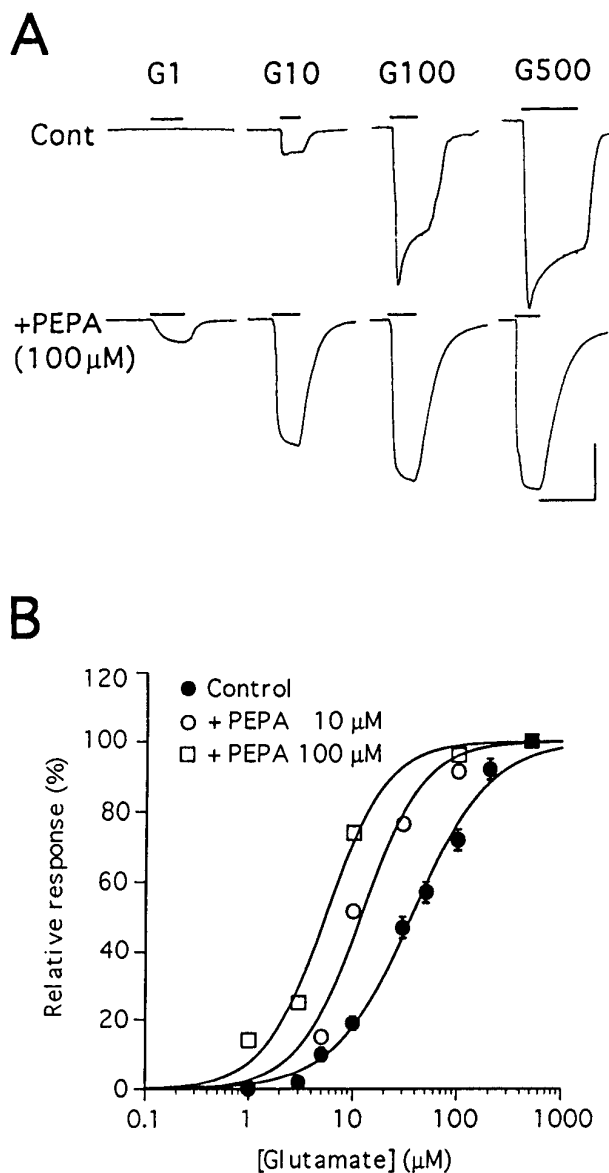


Figure 6. PEPA increases apparent affinity of GluR_C for glutamate. *A*, Responses to 1, 10, 100, and 500 μ M glutamate in the absence (Cont) or presence (+PEPA) of 100 μ M PEPA in oocytes expressing GluR_C. Calibration: 20 sec for all traces, and 20 nA for Cont and 250 nA for +PEPA. *B*, Dose-response curves for glutamate in oocytes expressing GluR_C without PEPA (Control) and with PEPA (10 or 100 μ M). Glutamate responses are normalized to the response by 500 μ M glutamate in each oocyte. The theoretical curves indicate the best fit to the data according to the logistic function $I = 100/[1 + (EC_{50}/[G])^n]$, in which I is the relative response, and $[G]$ is the concentration of glutamate. Values are mean \pm SEM ($n = 4-7$).

of 10 μ M PEPA, and $5 \pm 1 \mu$ M and 1.7 ($n = 4$) in the presence of 100 μ M PEPA. Thus although flop-selective, the effects of PEPA on AMPA receptor affinity for glutamate resemble those of cyclothiazide as opposed to aniracetam.

Weak potentiation by PEPA of kainate-evoked currents at AMPA receptors

Figure 7 compares the actions of PEPA (10 μ M) on glutamate-evoked (100 μ M) and kainate-evoked (100 μ M) currents in oocytes expressing the flip or flop splice variants of GluR_C. In oocytes expressing GluR_C, glutamate responses were much

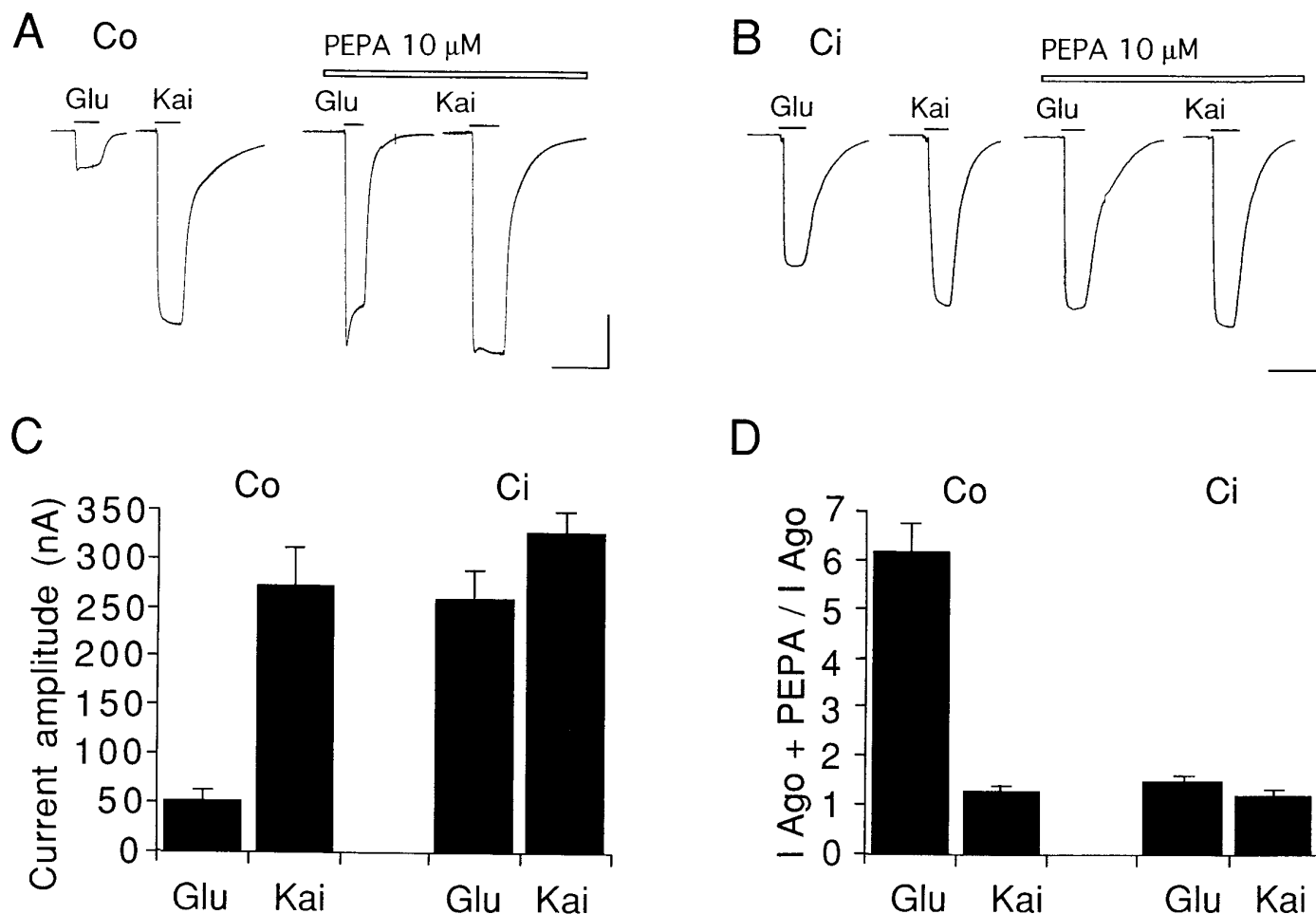


Figure 7. Activation of AMPA receptors by kainate shows weak potentiation by PEPA. Oocytes from a single preparation were injected with cRNA specific for GluRC_o or GluRC_i in parallel. *A, B*, Glutamate-evoked (100 μ M) and kainate-evoked (100 μ M) responses with or without PEPA (10 μ M) in oocytes expressing GluRC_o (*A*) or GluRC_i (*B*). Calibration, 20 sec and 100 nA. *C*, Comparison of the current amplitude (nA) for glutamate and kainate responses in oocytes expressing GluRC_o ($n = 10$) or GluRC_i ($n = 8$). *D*, Potentiation by 10 μ M PEPA of glutamate or kainate response in oocytes expressing GluRC_o ($n = 10$) or GluRC_i ($n = 7$). Values in *C* and *D* are mean \pm SEM.

smaller than kainate responses (Fig. 7*A,C*), such that the ratio of their amplitudes ($I_{\text{glu}}/I_{\text{kai}}$) was 0.19 ± 0.02 ($n = 10$). This low value is attributable in part to stronger desensitization of responses to glutamate versus kainate at AMPA receptors (Patneau and Mayer, 1991; Patneau et al., 1993), a difference that is enhanced for flip versus flip splice variants (Lomeli et al., 1994; Mosbacher et al., 1994; Partin et al., 1994). Consistent with this, in oocytes expressing GluRC_o the amplitude of glutamate responses (53 ± 11 nA) was much smaller than for oocytes expressing GluRC_i (258 ± 27 nA), although the amplitude of equilibrium responses to kainate was similar for GluRC_o (274 ± 36 nA) and GluRC_i (325 ± 22 nA). As a result, the $I_{\text{glu}}/I_{\text{kai}}$ ratio for GluRC_i (0.77 ± 0.04 , $n = 7$) was fourfold greater than for GluRC_o.

The difference in $I_{\text{glu}}/I_{\text{kai}}$ ratios for GluRC_i versus GluRC_o is of interest because PEPA potentiated glutamate responses for GluRC_o to a much greater extent than kainate responses (Fig. 7*A,D*), whereas for GluRC_i, although potentiation by PEPA was weaker than for GluRC_o, the extent of potentiation was similar for both agonists (Fig. 7*B,D*). A similar agonist dependence for potentiation of AMPA receptor responses by aniracetam and cyclothiazide has been observed previously (Tsuzuki et al., 1992; Partin et

al., 1994). These results suggest that PEPA potentiates AMPA receptor responses at least in part by reducing desensitization.

PEPA suppresses AMPA receptor desensitization in HEK 293 cells

A widely recognized limitation of the use of two-electrode voltage-clamp recording from oocytes is that it is impossible to apply solutions rapidly enough to resolve the very rapid desensitization exhibited by AMPA receptors. Analysis of the effects of drugs on AMPA receptor responses is confounded further by subunit and splice variant-specific differences in the amount of desensitization of control responses to glutamate, such that the amplitudes of equilibrium currents expressed by AMPA receptors vary with subunit composition (Lomeli et al., 1994; Mosbacher et al., 1994; Partin et al., 1994). One consequence of this would be that a receptor that normally desensitizes by 90% could be potentiated maximally only 10-fold by block of desensitization, whereas a receptor that normally desensitizes by 99% could be potentiated 100-fold. Thus, the apparent selectivity of PEPA for GluRC and GluRD might not reflect simply a higher affinity of PEPA for these subunits, because the inherently stronger desen-

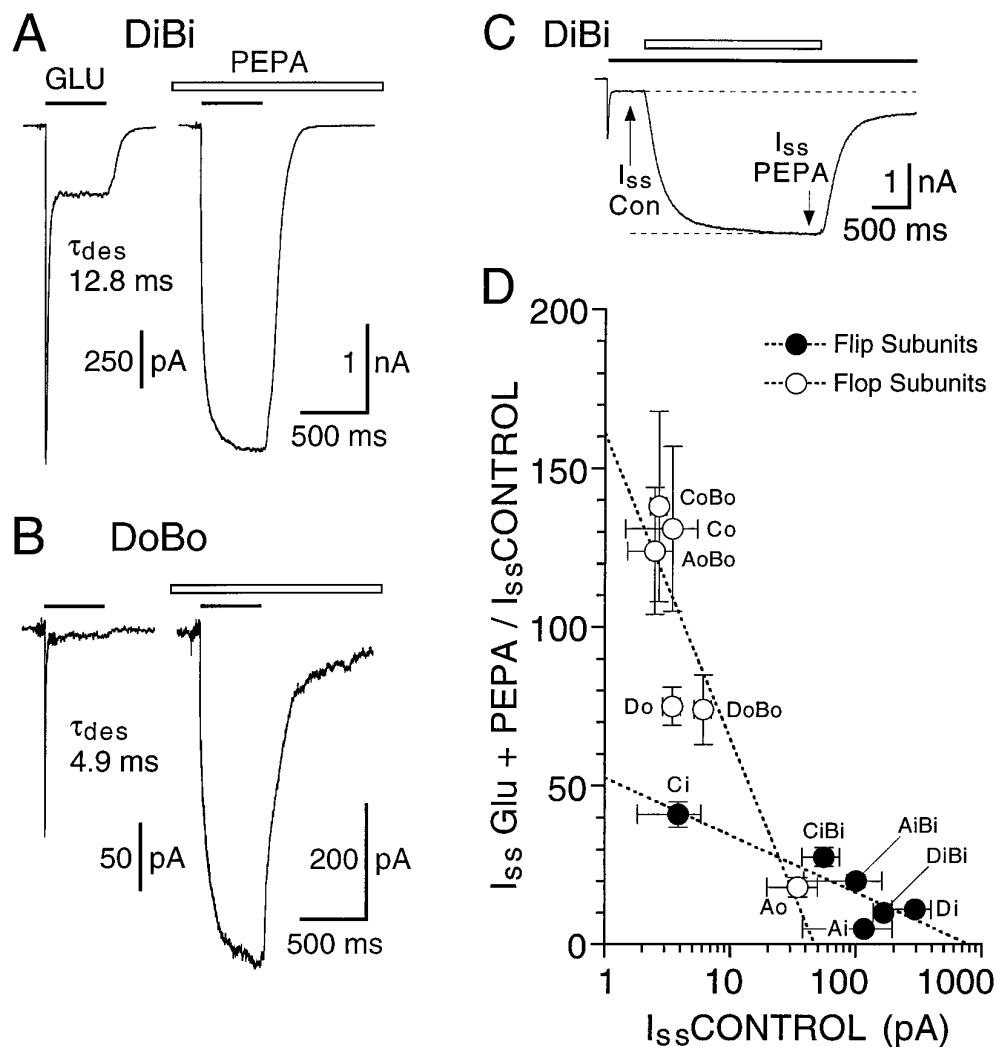


Figure 8. Potentiation by PEPA is correlated with desensitization of control responses to glutamate. *A*, Whole-cell responses to fast application of 3 mM glutamate in the absence (*left*) and presence (*right*) of 100 μ M PEPA recorded from HEK 293 cells transiently transfected with GluRB_iD_i; *B*, Similar responses for GluRB_oD_o. Control responses for GluRB_oD_o display faster desensitization, smaller equilibrium currents, and greater equilibrium potentiation by PEPA than responses for GluRB_iD_i. A similar correlation between the extent of control desensitization and potentiation by PEPA was observed for all flip and flop subunit combinations examined, although the extent of the block of desensitization by 100 μ M PEPA varied among subunits. *C*, Concentration jump responses to PEPA reveal rapid onset and recovery from potentiation. *D*, Scatterplot correlating equilibrium potentiation by 100 μ M PEPA versus the steady-state amplitude of control responses to 3 mM glutamate; data for flip (●) and flop (○) splice variants are fit separately with logarithmic functions (*dashed lines*; *points* are mean \pm SEM). Control responses to glutamate are smaller and potentiation by PEPA is greater for flop versus flip subunits. Heteromeric combinations of GluRA, GluRC, or GluRD with GluRB were transfected at a ratio of 1:2.

sitization for the flop splice variants of GluRC and GluRD (Lomeli et al., 1994; Mosbacher et al., 1994; Partin et al., 1994) also could increase potentiation of equilibrium responses to glutamate if the mechanism underlying potentiation by PEPA involved block of desensitization.

To address this issue, we used whole-cell recording with rapid perfusion to study recombinant AMPA receptor responses in transiently transfected HEK 293 cells. The kinetics of AMPA receptor desensitization was measured in response to concentration jump application of 3 mM glutamate in the absence or presence of 100 μ M PEPA (Fig. 8*A,B*). We also measured potentiation of equilibrium responses to glutamate in response to concentration jump application of PEPA (Fig. 8*C*). The combined results of such analysis reveal that, for subunit combinations for which PEPA fully blocks desensitization, differences in the extent of desensitization of control responses to glutamate indeed do appear to contribute to the extent of potentiation by PEPA. For example, although PEPA produced nearly complete block of desensitization for both GluRB_oD_o and GluRB_iD_i (Fig. 8*A,B*), potentiation of equilibrium responses to glutamate for these subunits suggested strong flop selectivity (GluRB_oD_o, 74 ± 10.8 -fold potentiation; GluRB_iD_i, 9.7 ± 1.2 -fold potentiation). Experiments with additional subunits provided further support for the hypothesis that the degree of potentiation by PEPA is determined

in part by the extent of desensitization of control responses to glutamate; thus, as shown in Figure 8*D*, the magnitude of potentiation by PEPA was related inversely to the amplitude of equilibrium responses to glutamate in the absence of PEPA. Subunit combinations for which the onset of desensitization has been shown to occur extremely rapidly—GluRC_o, GluRD_o, GluRB_oC_o, and GluRB_oD_o (Mosbacher et al., 1994)—and that show the greatest extent of desensitization at equilibrium (Lomeli et al., 1994) showed much stronger potentiation by PEPA than did GluRA_i, GluRD_i, GluRA_iB_i, and GluRB_iD_i subunit combinations for which the onset of desensitization is relatively slow and that show less desensitization of control responses to glutamate (Lomeli et al., 1994; Mosbacher et al., 1994). The greater extent of potentiation by PEPA in HEK 293 cells (Fig. 8*D*) than in oocytes (see Fig. 4) possibly reflects difficulties in accurately measuring the equilibrium amplitude of control responses to glutamate in HEK 293 cells transfected with strongly desensitizing subunits. In *Xenopus* oocytes we noticed that the extent of potentiation by PEPA for GluRC_o and GluRD_o was related inversely to the amplitude of control responses to glutamate, possibly also reflecting inaccuracy in measuring the amplitude of control responses for these strongly desensitizing subunits.

The results shown in Figure 8 suggest that PEPA directly affects the process of desensitization and that subunit-specific

Table 1. AMPA receptor kinetics in transfected HEK 293 cells

| Subunit(s) | τ_{des} (Glu) | τ_{des} (Glu+PEPA) | Desensitization at 2 sec (%) (Glu+PEPA) | Slowing of desensitization $\tau(Glu+P)/\tau(Glu)$ | Equilibrium potentiation $I(Glu+P)/I(Glu)$ | PEPA τ_{ON} | PEPA τ_{OFF} fast component | τ_{OFF} % fast |
|-------------------------------|----------------------|-------------------------|---|--|--|------------------|----------------------------------|---------------------|
| A _i | 7.98 ± 0.48 | 89 ± 6 | 84 ± 3 | 11.2 | 5 ± 1 | 122 ± 14 | 87 ± 7 | 87 |
| A _i B _i | 10.43 ± 0.70 | 244 ± 19 | 28 ± 3 | 23.4 | 20 ± 2 | 306 ± 17 | 140 ± 8 | 84 |
| A _o | 8.04 ± 0.69 | 252 ± 13 | 74 ± 6 | 31.3 | 18 ± 3 | 338 ± 28 | 176 ± 15 | 88 |
| A _o B _o | 6.43 ± 0.45 | N.D. | 1 ± 1 | >300** | 124 ± 20 | 521 ± 39 | 222 ± 15 | 79 |
| C _i | 9.92 ± 1.20 | 300 ± 25 | 45 ± 4 | 30.2 | 41 ± 4 | 253 ± 12 | 156 ± 20 | 85 |
| B _i C _i | 14.40 ± 0.76 | N.D. | 6 ± 2 | >140** | 28 ± 3 | 257 ± 24 | 153 ± 8 | 93 |
| C _o | 3.28 ± 0.34 | 314 ± 18 | 47 ± 4 | 95.7 | 131 ± 26 | 308 ± 21 | 193 ± 16 | 87 |
| B _o C _o | 4.39 ± 0.38 | 564 ± 45* | 15 ± 2* | >300** | 138 ± 30 | 475 ± 25 | 226 ± 10 | 89 |
| D _i | 9.15 ± 0.56 | 365 ± 22 | 28 ± 5 | 39.9 | 11 ± 1 | 372 ± 27 | 201 ± 9 | 65 |
| B _i D _i | 14.15 ± 1.09 | N.D. | 4 ± 3 | >140** | 10 ± 1 | 335 ± 47 | 192 ± 11 | 73 |
| D _o | 3.11 ± (n = 2 of 9)# | N.D. | 7 ± 1 | >600** | 75 ± 6 | 417 ± 29 | 237 ± 9 | 85 |
| B _o D _o | 6.46 ± 0.65 | N.D. | 5 ± 1 | >300** | 74 ± 11 | 571 ± 50 | 267 ± 11 | 70 |

Values are mean ± SEM, in msec, for 3–20 cells per subunit. Examples illustrating analysis of τ_{des} (Glu) and τ_{des} (Glu+PEPA) are shown in Figures 8A and 9A; analysis of equilibrium potentiation and the rate of onset (τ_{ON}) and recovery (τ_{OFF}) from potentiation by PEPA are shown in Figure 8B. *Indicates that 14 of 20 cells expressing GluRB_oC_o desensitized by 20.4% with a mean time constant of 564 msec, whereas six other cells desensitized by only 3.7%. **Estimated slowing of $\tau(G+P)/\tau(G)$ assumes a value of 2 sec for τ_{des} (Glu+PEPA) in the case of subunit combinations for which desensitization in the presence of PEPA was either too slow or too small to perform the exponential analysis accurately. #Indicates that for only two of nine cells were control responses to glutamate large enough to estimate τ_{des} accurately.

differences in the magnitude of PEPA potentiation are likely to reflect both the intrinsic desensitization properties of individual subunits as well as possible differences in affinity for PEPA. Although for some subunits (e.g., GluRC_o and GluRD_o) the rate of desensitization of control responses to glutamate was too rapid to be measured accurately by whole-cell recording, for all subunit combinations tested both the rate of onset and amount of desensitization were strongly attenuated by PEPA, making it relatively easy to compare differences in desensitization kinetics among subunits in the presence of PEPA (Table 1).

The results of such an analysis are shown in Figure 9 and emphasize both the selective modulation by PEPA of AMPA receptor flop splice variants as well as subunit-selective effects of PEPA. Thus, even for GluRA, the least sensitive subunit examined for which desensitization remained pronounced in the presence of PEPA for both flip and flop splice variants (Fig. 9A), the time constant of onset of desensitization for GluRA_o ($\tau_{control}$, 8.0 ± 0.4 msec; τ_{PEPA} , 252 ± 13 msec; n = 14) was slowed by 100 μ M PEPA nearly three times more than for GluRA_i ($\tau_{control}$, 8.0 ± 0.5 msec; τ_{PEPA} , 89 ± 6 msec; n = 11). In addition, a residual fast-desensitizing component of time constant identical to that for control responses to glutamate and that accounted for 27 ± 4% of the decay in the presence of 100 μ M PEPA suggests that for GluRA_i the binding of PEPA is not saturated at 100 μ M, whereas for GluRA_o desensitization in the presence of 100 μ M PEPA was well fit by a single exponential, suggesting that binding of PEPA to GluRA_o is saturated at 100 μ M. Although the flop-selective action of PEPA is indicated further by the complete block of desensitization by 100 μ M PEPA for GluRD_o and GluRA_oB_o, but not GluRD_i and GluRA_iB_i (Table 1 and Fig. 9B), for other subunits (GluRC_o vs GluRC_i and GluRB_oD_o vs GluRB_iD_i) there was no clear difference in the extent of attenuation of desensitization by 100 μ M PEPA between flip and flop splice variants; in contrast to the similar degree of equilibrium potentiation for these subunits (Table 1), the subunit dependence for slowing of desensitization followed the rank order GluRD > GluRC >> GluRA (Fig. 9).

The effects of PEPA were enhanced further by heteromerization with the corresponding flip or flop isoforms of GluRB, and

the current observed after a 2 sec application of GLU+PEPA approached 100% of peak in cells expressing GluRA_oB_o, GluRB_iC_i, GluRB_iD_i, and GluRB_oD_o; these subunit combinations, together with GluRD_o, therefore were classified as effectively “nondesensitizing” within the limits of the current protocol (Fig. 9B). In an attempt to differentiate among subunits that effectively were nondesensitizing during 2 sec applications of PEPA+GLU, we also measured the kinetics of onset of (τ_{on}) and recovery from (τ_{off}) potentiation by PEPA in the continuous presence of glutamate (Fig. 8C, Table 1). Such experiments revealed responses to PEPA with kinetics more complex than that predicted by a simple model in which binding of PEPA blocks desensitization, because the response to removal of PEPA frequently showed both fast and slow components of decay of potentiation (Fig. 8C). In addition, for flop subunits responses to glutamate in the presence of PEPA tended to show fast and slow components of activation and deactivation (Figs. 8B, 9A), indicating that the mechanism or mechanisms of action of PEPA are likely to be complex. Within these limitations and assuming that τ_{off} approximates the inverse rate constant for dissociation of PEPA and that the rate constant for binding of PEPA does not differ among subunits, analysis that uses the protocol shown in Figure 8C gives an indirect measure of subunit-dependent differences in affinity for PEPA (Table 1). In cells expressing AMPA receptor flop isoforms, values for τ_{off} in response to removal of PEPA were consistently slower than for the corresponding flip isoforms, suggesting a higher affinity of flop receptors for PEPA. However, between the fastest (GluRA_i; τ_{off} , 87 ± 7 msec) and slowest responding subunits (GluRD_o; τ_{off} , 237 ± 9 msec) there was only a threefold range in kinetics of recovery from potentiation, indicating that additional mechanisms must underlie subunit selective modulation by PEPA. Especially interesting is the comparison for responses to 100 μ M cyclothiazide recorded with similar protocols (Patneau et al., 1993; Partin et al., 1994), which reveal that the onset of potentiation by 100 μ M PEPA develops ~50–100 times faster than for cyclothiazide, although both drugs produce nearly complete block of desensitization for selected subunit combinations. The kinetics of recovery from potentiation by PEPA (Table 1) was also much

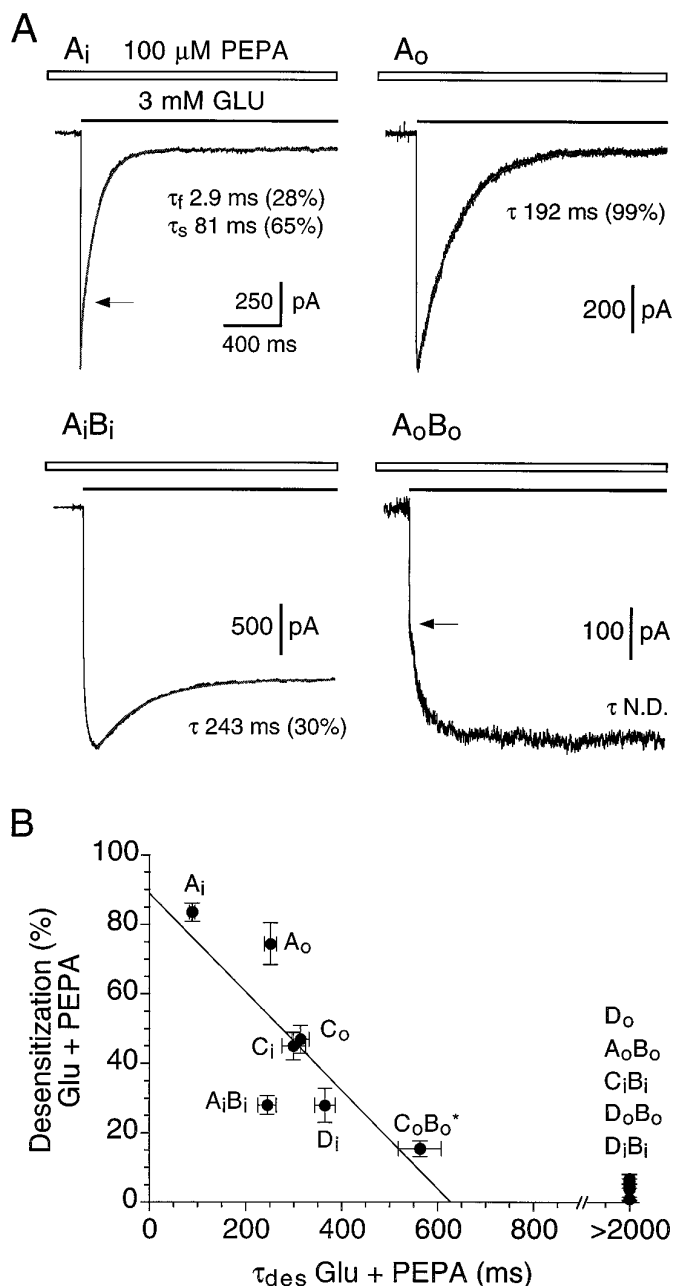


Figure 9. Block of desensitization by PEPA shows subunit and splice-variant selectivity. *A*, Whole-cell responses to fast application of 3 mM glutamate applied in the presence of 100 μ M PEPA and recorded from transiently transfected HEK 293 cells expressing GluRA_i, GluRA_o, GluRA_iB_i, or GluRA_oB_o, as indicated. Lines drawn through the data points show the onset of desensitization fit with exponential functions, as described in Materials and Methods. For GluRA_i and GluRA_oB_o, arrows indicate transitions between fast and slow components of the response to glutamate; the slow component of activation seen for GluRA_oB_o was typical of responses for subunit combinations strongly modulated by PEPA. Values for τ_{des} (mean \pm SEM, $n = 3$ –14 cells) were 89 \pm 6 msec (GluRA_i), 252 \pm 13 msec (GluRA_o), and 244 \pm 19 msec (GluRA_iB_i). *B*, Summary of results from transfections with additional AMPA receptor flip and flop splice variants for which the extent and rate of onset of desensitization in the presence of 100 μ M PEPA were estimated as shown in *A*. Star indicates that for GluRC_oB_o only 14 of 20 cells gave sufficiently strongly desensitizing responses to estimate the rate of onset of desensitization; for GluRD_o, GluRA_oB_o, GluRC_iB_i, and GluRD_oB_o, which gave weakly or nondesensitizing responses for which it was not possible to fit exponentials accurately, the time constant of desensitization is given as >2000 msec.

faster than that for recovery from potentiation by cyclothiazide (Patneau et al., 1993; Partin et al., 1994).

DISCUSSION

Our results show that PEPA is a selective and potent modulator of AMPA subtype glutamate receptors, with preferential activity at flop splice variants. PEPA potentiates glutamate-evoked currents by slowing the rate of onset of desensitization and by increasing the apparent affinity of AMPA receptors for agonist. It is possible that PEPA also slows deactivation, but this was not tested in the present experiments. The effects of PEPA were much stronger for GluRC and GluRD versus GluRA, most likely reflecting either subunit-dependent differences in affinity and/or stronger desensitization of control responses to glutamate for GluRC and GluRD versus GluRA.

PEPA versus aniracetam

Comparison with previous work (Johansen et al., 1995; Partin et al., 1996) suggests that PEPA and aniracetam have similar subunit and splice-variant selectivity; however, PEPA is considerably more potent than aniracetam in potentiating AMPA receptor currents. In agreement with this, concentrations in excess of 1–5 mM aniracetam are required for strong potentiation of AMPA receptor currents in oocytes (Tsuzuki et al., 1992; Johansen et al., 1995); in fact, saturation of the dose–response relationship cannot be achieved because of the low affinity and limited solubility of aniracetam in physiological solutions. In contrast, PEPA effectively potentiates AMPA receptor responses at 10–200 μ M concentrations. It was reported that 1 mM aniracetam potentiates glutamate-evoked currents \sim 2.5-fold in oocytes expressing GluRA_o (Tsuzuki et al., 1992), whereas a fivefold lower concentration of PEPA (200 μ M) potentiates GluRA_o responses eightfold (Fig. 4). Another study reported eightfold potentiation by 5 mM aniracetam for oocytes expressing GluRA_iB_i or GluRB_iD_i, 17-fold potentiation for GluRA_oB_o, and 77-fold potentiation for GluRB_oD_o (Johansen et al., 1995); we have found comparable amounts of potentiation by 100–200 μ M PEPA in the present study. Another remarkable difference between PEPA and aniracetam is their apparent efficacy for block of desensitization. Although limited solubility makes it impossible to assess the maximal effect of aniracetam, PEPA appears to have a much greater effect on slowing desensitization. In rapid perfusion experiments on HEK 293 cells expressing GluRA_i or GluRA_o, the rate of onset of desensitization (τ_{des}) in the presence of 5 mM aniracetam, 20 and 32 msec, respectively (Partin et al., 1996), was much faster than in the presence of 100 μ M PEPA, 89 and 252 msec, respectively (Fig. 9).

PEPA versus cyclothiazide

In the present study PEPA (25 μ M) was found to potentiate glutamate responses for GluRC_o to a much greater extent than the same concentration of cyclothiazide, whereas the reverse was true for GluRC_i (see Fig. 2C). PEPA and cyclothiazide appear to have similar potency but opposite splice-variant selectivity; whereas PEPA preferentially modulates flop receptors, cyclothiazide has been shown to prefer flip isoforms (Partin et al., 1994). However, in rapid perfusion experiments on HEK 293 cells expressing GluRC, the extent and rate of onset of desensitization of responses to glutamate in the presence of 100 μ M PEPA were similar for GluRC_o (τ_{des} , 314 \pm 18 msec) and GluRC_i (τ_{des} ,

300 ± 25 msec), indicating that the flop selectivity of PEPA for potentiation of GluRC equilibrium responses to glutamate most likely reflects differences in the extent of desensitization of control responses to glutamate. In contrast, for GluRA_o the time constant of desensitization in the presence of 100 μM PEPA (252 ± 13 msec) was much slower than for GluRA_i (τ_{des}, 89 ± 6 msec), whereas for 100 μM cyclothiazide desensitization was blocked almost fully for GluRA_i but remained pronounced for GluRA_o (Partin et al., 1994). The flop selectivity for modulation of desensitization by PEPA was modified by coassembly with GluRB; thus, almost complete block of desensitization by 100 μM PEPA was observed for the flop splice-variant combinations GluRB_oC_o and GluRB_oD_o but also for the corresponding flip splice-variant combinations GluRB_iC_i and GluRB_iD_i. For GluRA_i and GluRA_o, coassembly with the corresponding flip and flop splice variants of GluRB also increased the extent of block of desensitization by PEPA, although the effect was greater for GluRA_oB_o than for GluRA_iB_i (Fig. 9).

Mechanism of action of PEPA

A characteristic feature of allosteric modulation by PEPA, which is common also to aniracetam and cyclothiazide, is that each drug potentiates AMPA receptor currents by suppressing desensitization. However, it has been proposed recently that aniracetam and cyclothiazide suppress desensitization by different mechanisms (Partin et al., 1996). Kinetic modeling of the rapid deactivation and desensitization kinetics of GluRA_i and GluRA_o suggests that aniracetam slows the rate of channel closing, indirectly slowing the onset of desensitization, whereas cyclothiazide has a direct effect on the rate constant of desensitization and, in addition, stabilizes agonist-bound closed states, thus increasing agonist affinity and slowing deactivation. Whether this model is sufficient to explain the behavior of other subunits or of heteromeric subunit combinations remains to be determined. Although additional experiments are required to develop a kinetic model for the action of PEPA, our results show that PEPA does not behave exactly like aniracetam, because for some subunits PEPA completely prevented desensitization. If this were attributable exclusively to slowing of deactivation, as modeled for aniracetam modulation of GluRA (Partin et al., 1996), we would expect a considerably more pronounced slowing of the current decay after removal of agonist than was observed in our experiments (Fig. 8). Also, PEPA caused a leftward shift in the agonist dose–response relationship for GluRC_o (Fig. 6) reminiscent of the action of cyclothiazide, but not aniracetam. A detailed analysis of deactivation kinetics in the presence of PEPA and experiments designed to resolve the origin of the slow onset of responses to glutamate in the presence of PEPA (Figs. 8, 9) will be necessary to understand how modulation by PEPA differs from that produced by aniracetam and cyclothiazide.

Drug design and receptor structure–function analysis

When the chemical structures are compared among aniracetam, cyclothiazide, and PEPA (see Fig. 1), no obvious features common to all three drugs are seen. There is, however, a sulfonylamino group (-SO₂NH-) common to PEPA, cyclothiazide, and other benzothiadiazines that has been shown to suppress AMPA receptor desensitization (Bertolino et al., 1993; Yamada and Tang, 1993). The fact that this functional group is not found in aniracetam suggests that it might be important for coupling drug

binding to modulation of desensitization versus channel-gating kinetics.

Although the actions of aniracetam and cyclothiazide are different, it has been shown previously that modulation by both drugs is sensitive to a single amino acid residue in the flip/flop domain (Partin et al., 1995). This position contains a serine residue in flip splice variants, an asparagine residue in flop splice variants, and a glutamine residue at the equivalent site in cyclothiazide-insensitive kainate receptors (S/N/Q site). Exchange of the serine and asparagine residues by site-directed mutagenesis (GluRA_iS750N and GluRA_oN750S) is sufficient to exchange flip-like and flop-like modulation by aniracetam and cyclothiazide (Partin et al., 1995, 1996). Preliminary observations in HEK 293 cells expressing GluRA_iS750N and in oocytes expressing the corresponding GluRC_i mutant S758N confirm that this site also is involved in the action of PEPA and that the exchange of serine for asparagine is sufficient to convert flip-like to flop-like modulation (data not shown). Furthermore, in HEK 293 cells sensitivity to cyclothiazide, but not PEPA or aniracetam, is abolished for the mutant GluRA_iS750Q. It remains to be determined, however, whether the S/N/Q site contributes to a binding site for these drugs or is involved in directing a conformational transition on which all three drugs converge.

Recently, a three-dimensional model for non-NMDA glutamate receptors has been proposed (Sutcliffe et al., 1996). This model proposes a disulfide bond linking two highly conserved Cys residues, which are located in a large extracellular loop between the M3 and M4 domains (Hollmann et al., 1994; Stern-Bach et al., 1994; Bennett and Dingledine, 1995). One of these Cys residues is located in the S2 domain, one of two putative agonist-binding domains that show structural homology with bacterial periplasmic lysine/arginine/ornithine binding protein (LAOBP; Stern-Bach et al., 1994). The other is located in the flip/flop segment, which is implicated in deactivation and desensitization (Mosbacher et al., 1994; Partin et al., 1996). Formation of a disulfide bond between these residues may be important for the actions of cyclothiazide and PEPA, because these drugs affect apparent affinity for both agonist and channel kinetics.

Pharmacological and physiological significance of allosteric modulation

Because of its strong preference for flip isoforms, cyclothiazide has been used to infer the splice-variant composition of native AMPA receptors in subpopulations of hippocampal neurons (Fleck et al., 1996). Cyclothiazide also has been useful in examining the relationship between desensitization and synaptic efficacy. Similarities in the rate of AMPA receptor desensitization and the rate of synaptic current decay in certain neuronal populations suggest that at some synapses the rapid onset of AMPA receptor desensitization may serve to limit the duration of the excitatory postsynaptic response and/or induce postsynaptic depression (Trussell and Fischbach, 1989; Otis et al., 1996). However, although both cyclothiazide (Trussell et al., 1993; Yamada and Tang, 1993; Raman et al., 1994) and aniracetam (Isaacson and Nicoll, 1991; Tang et al., 1991; Vycklicky et al., 1991; Hestrin, 1992) have been shown to enhance the peak amplitude and prolong the duration of excitatory postsynaptic currents, at many synapses this most likely results from a combination of presynaptic effects and a reduced rate of channel closing rather than from block of desensitization (Hestrin, 1992; Diamond and Jahr, 1995). For this reason further work to establish the mechanism or

mechanisms by which PEPA modulates AMPA receptors will be required before it would be wise to use this drug to investigate the role of AMPA receptor subtypes in synaptic transmission. In addition, it should be noted that, because PEPA was synthesized in the process of developing thromboxane A2 antagonists, it is possible that it could act on targets distinct from glutamate receptors; we established that PEPA is selective for AMPA versus kainate and NMDA receptors, but its effects on other receptor species have not yet been investigated.

Other studies have suggested that the memory- and cognition-enhancing properties of the pyrrolidinone- and benzothiadiazine-containing drugs are related to their ability to enhance synaptic transmission via modulation of AMPA receptor gating (Arai et al., 1994; Staubli et al., 1994a,b; Johansen et al., 1995; Zivkovic et al., 1995). In this regard, PEPA may be useful as a pharmacological tool and could provide new clues in the search for novel and more potent drugs affecting memory and cognition.

REFERENCES

- Arai A, Kessler M, Xiao P, Ambros-Ingerson J, Rogers G, Lynch G (1994) A centrally active drug that modulates AMPA receptor gated currents. *Brain Res* 638:343-346.
- Bennett JA, Dingledine R (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining re-entrant membrane loop. *Neuron* 14:373-384.
- Bertolino M, Baraldi M, Parenti C, Braghiroli D, DiBella M, Vicini S, Costa E (1993) Modulation of AMPA/kainate receptors by analogues of diazoxide and cyclothiazide in thin slices of rat hippocampus. *Receptors Channels* 1:267-278.
- Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* 249:1033-1037.
- Bowie D, Mayer ML (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* 15:453-462.
- Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745-2752.
- Diamond JS, Jahr CE (1995) Asynchronous release of synaptic vesicles determines the time course of the AMPA receptor-mediated EPSC. *Neuron* 15:1097-1107.
- Ferrer-Montiel AV, Montal M (1996) Pentameric subunit stoichiometry of a neuronal glutamate receptor. *Proc Natl Acad Sci USA* 93:2741-2744.
- Fleck MW, Bähring R, Patneau DK, Mayer ML (1996) AMPA receptor heterogeneity in rat hippocampal neurons revealed by differential sensitivity to cyclothiazide. *J Neurophysiol* 75:2322-2333.
- Hestrin S (1992) Activation and desensitization of glutamate-activated channels mediating fast excitatory synaptic currents in the visual cortex. *Neuron* 9:991-999.
- Hollmann M, O'Shea-Greenfield A, Rogers SW, Heinemann S (1989) Cloning by functional expression of a member of the glutamate receptor family. *Nature* 342:643-648.
- Hollmann M, Hartley M, Heinemann S (1991) Ca²⁺ permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 252:851-853.
- Hollmann M, Maron C, Heinemann S (1994) N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. *Neuron* 13:1331-1343.
- Hume RI, Dingledine R, Heinemann SF (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* 253:1028-1031.
- Isaacson JS, Nicoll RA (1991) Aniracetam reduces glutamate receptor desensitization and slows the decay of fast excitatory synaptic currents in the hippocampus. *Proc Natl Acad Sci USA* 88:10936-10940.
- Ito I, Tanabe S, Kohda A, Sugiyama H (1990) Allosteric potentiation of quisqualate receptors by a nootropic drug aniracetam. *J Physiol (Lond)* 424:533-543.
- Johansen TH, Chaudhary A, Verdoorn TA (1995) Interactions among GYKI-52466, cyclothiazide, and aniracetam at recombinant AMPA and kainate receptors. *Mol Pharmacol* 48:946-955.
- Keinänen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. *Science* 249:556-560.
- Lomeli H, Mosbacher J, Melcher T, Höger T, Geiger JRP, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266:1709-1713.
- Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, Wada K (1995) Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J Neurochem* 65:454-458.
- Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. *Neuron* 6:799-810.
- Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg P, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266:1059-1062.
- Otis T, Zhang S, Trussell LO (1996) Direct measurement of AMPA receptor desensitization induced by glutamatergic synaptic transmission. *J Neurosci* 16:7496-7504.
- Partin KM, Patneau DK, Mayer ML (1994) Cyclothiazide differentially modulates desensitization of AMPA receptor splice variants. *Mol Pharmacol* 46:129-138.
- Partin KM, Bowie D, Mayer ML (1995) Structural determinants of allosteric regulation in alternatively spliced AMPA receptors. *Neuron* 14:833-843.
- Partin KM, Fleck MW, Mayer ML (1996) AMPA receptor flip/flop mutants affecting deactivation, desensitization, and modulation by cyclothiazide, aniracetam, and thiocyanate. *J Neurosci* 16:6634-6647.
- Patneau DK, Mayer ML (1991) Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. *Neuron* 6:785-798.
- Patneau DK, Vyklicky L, Mayer ML (1993) Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. *J Neurosci* 13:3496-3509.
- Raman IM, Zhang S, Trussell LO (1994) Pathway-specific variants of AMPA receptors and their contribution to neuronal signaling. *J Neurosci* 14:4998-5010.
- Sato M, Kawashima Y, Goto J, Yamane Y, Chiba Y, Jinno S, Satake M, Iwata C (1994) Synthesis and evaluation of novel sulfonamide derivatives as thromboxane A2 receptor antagonists. *Eur J Med Chem* 29:185-190.
- Sato M, Kawashima Y, Goto J, Yamane Y, Chiba Y, Jinno S, Satake M, Iwata C (1995) Synthesis and evaluation of novel fluorinated sulotroban-related sulfonamide derivatives as thromboxane A2 receptor antagonists. *Eur J Med Chem* 30:403-414.
- Sekiguchi M, Doi K, Zhu WS, Watase K, Yokotani N, Wada K, Wenthold RJ (1994) A deletion in the second cytoplasmic loop of GluR3 produces a dominant negative mutant of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor. *J Biol Chem* 269:14559-14565.
- Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Köhler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* 249:1580-1585.
- Sommer B, Köhler M, Sprengel R, Seeburg PH (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67:11-19.
- Staubli U, Perez Y, Xu FB, Rogers G, Ingvar M, Stone-Elender S, Lynch G (1994a) Centrally active modulators of glutamate receptors facilitate the induction of long-term potentiation *in vivo*. *Proc Natl Acad Sci USA* 91:11158-11162.
- Staubli U, Rogers G, Lynch G (1994b) Facilitation of glutamate receptors enhances memory. *Proc Natl Acad Sci USA* 91:777-781.
- Stein E, Cox JA, Seeburg PH, Verdoorn TA (1992) Complex pharmacological properties of recombinant alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtypes. *Mol Pharmacol* 42:864-871.
- Stern-Bach Y, Bettler B, Hartley M, Sheppard PO, O'Hara PJ, Heinemann SF (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid binding proteins. *Neuron* 13:1345-1357.
- Sutcliffe M, Wo ZG, Oswald RE (1996) Three-dimensional models of non-NMDA glutamate receptors. *Biophys J* 70:1575-1589.
- Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. *J Neurosci* 17:58-69.

- Tang CM, Shi QY, Katchman A, Lynch G (1991) Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. *Science* 254:288–290.
- Trussell LO, Fischbach GD (1989) Glutamate receptor desensitization and its role in synaptic transmission. *Neuron* 3:209–218.
- Trussell LO, Zhang S, Raman IM (1993) Desensitization of AMPA receptors upon multiquantal neurotransmitter release. *Neuron* 10:1185–1196.
- Tsuzuki K, Takeuchi T, Ozawa S (1992) Agonist- and subunit-dependent potentiation of glutamate receptors by a nootropic drug aniracetam. *Mol Brain Res* 16:105–110.
- Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 252:1715–1718.
- Vyklicky L, Patneau DK, Mayer ML (1991) Modulation of excitatory synaptic transmission by drugs that reduce desensitization at AMPA/kainate receptors. *Neuron* 7:971–984.
- Wenthold RJ, Yokotani N, Doi K, Wada K (1992) Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies. Evidence for a hetero-oligomeric structure in rat brain. *J Biol Chem* 267:501–507.
- Yamada KA, Rothman SM (1992) Diazoxide reversibly blocks glutamate desensitization and prolongs excitatory postsynaptic currents in rat hippocampal neurons. *J Physiol (Lond)* 458:385–407.
- Yamada KA, Tang CM (1993) Benzothiadiazines inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *J Neurosci* 13:3904–3915.
- Zivkovic D, Thompson DM, Bertolino M, DiBella M, Costa E, Guidotti A (1995) 7-Chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine *S,S*-dioxide (IDRA21): a benzothiadiazine derivative that enhances cognition by attenuating DL-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) receptor desensitization. *J Pharmacol Exp Ther* 272:300–309.