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A desensitization-selective potentiator of AMPA-type glutamate receptors

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1 We examined the effects of PEPA, an allosteric potentiator of AMPA receptors, on AMPA receptor kinetics.

2 PEPA did not affect the deactivation of glutamate responses but potently attenuated the extent of receptor desensitization without slowing the onset of desensitization in most of the recombinant AMPA receptors (GluR1-flip, GluR1-flop, GluR3-flip, GluR3-flip + GluR2-flip, and GluR3-flop + GluR2-flop) expressed in *Xenopus* oocytes. For the GluR3-flop subunit, PEPA attenuated the extent of desensitization and only weakly prolonged deactivation (1.3 fold).

3 PEPA did not significantly affect recovery from desensitization in oocytes expressing GluR3-flip, GluR1-flop, and GluR1-flop, but weakly accelerated (2.6 fold) recovery from desensitization in oocytes expressing GluR3-flop.

4 PEPA's effect on desensitization of GluR3-flop-containing receptors is unique in that onset is very slow.

5 Simulation studies using simplified kinetic models for AMPA receptors are utilized to explore the differential effects of PEPA on GluR3-flip and -flop. It is possible to simulate the action on GluR3-flip by modulating two rate constants in a 12-state kinetic model. For simulation of the action on GluR3-flop, the 12-state kinetic model is not enough, and it is necessary to invoke a 13th state, a PEPA-bound receptor to which glutamate cannot bind.

6 These results suggest that attenuation of extent of desensitization represents the principal mechanism underlying the potentiation of AMPA receptors by PEPA, and that PEPA exhibits different mechanisms with respect to GluR3-flip and GluR3-flop.

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Abbreviations: AMPA: DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, PEPA: 4-[2-(phenylsulphonylamino)ethylthio]-2,6-difluorophenoxyacetamide

Introduction

Allosteric potentiators of AMPA receptors have gained special consideration since the finding that some potentiators improve performance of rats in the Morris water maze (Staubli *et al*., 1994), delayed recall in aged humans (Lynch *et al*., 1997), and delayed-nonmatch-to-sample performance in rats (Hampson *et al*., 1998a, b). It is also reported that some potentiators ameliorate hypercapnia- or scopolamine-induced cognitive impairment in rodents (Cumin *et al*., 1982) and alprazolam-induced learning deficit in patas monkeys working in a complex behavioural task (Thompson *et al*., 1995).

Currently, these allosteric potentiators are divided into three groups according to structure: the benzoylpiperidine-related potentiators (e.g., aniracetam, ampakines such as 1-BCP, CX-516), the thiazide-related potentiators (e.g., cyclothiazide, IDRA-21; reviewed in Yamada, 1998) and PEPA (4-[2-(phenylsulphonylamino)ethylthio]-2,6-difluorophenoxyacetamide; Sekiguchi *et al*., 1997; 1998). The action of some AMPA potentiators on receptor kinetics, deactivation, desensitization, and recovery from desensitization, has been studied in detail. Namely, aniracetam (Partin *et al*., 1996) and BDP-20 (Arai *et al*., 1996a) modulate both deactivation and desensitization kinetics, as do other benzoylpyrrolidine drugs BDP-12 (CX516; Arai *et al*., 1996b) and CX614 (Arai *et al*., 2000) in hippocampal pyramidal cells. Cyclothiazide modulates both types of kinetics in AMPA receptors containing the GluR1-flip splice variant (Partin *et al*., 1996), and the thiazide-related drug IDRA21 acts similarly in hippocampal neurons (Yamada *et al*., 1998). Such information is prerequisite to the formulation of a model of potentiator action with respect to molecular/cellular events or animal behaviour, since the deactivation and desensitization are the different processes that terminate glutamate response at AMPA receptors. For example, Vanover (1997) suggested that AMPA receptor modulators might not be uniform in their effects on dopamine-mediated behaviours and their antipsychotic potential. Such non-uniformity may result from differential effects of potentiators on receptor kinetics.

We previously found that PEPA potentiates AMPA receptor activity and modulates receptor desensitization (Sekiguchi *et al*., 1997). Still, a detailed understanding of the kinetics of PEPA's action is lacking. Such information is of great importance in light of the recent finding that PEPA ameliorates ischemia-induced impairment of performance of

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rats in the Morris water maze test (Sekiguchi *et al*., 2001). As mentioned above, AMPA receptor potentiators are suggested not to uniform with respect to their effects on dopamine-mediated behaviours and their antipsychotic potential. Thus, defining the kinetic basis by which receptors are modulated must precede our understanding of the action of AMPA potentiators on behaviour. Further, the use of a combination of cyclothiazide (affects flip-splice variants preferentially; Partin *et al*., 1994) and PEPA (affects flop-splice variants preferentially) could enable the characterization of AMPA receptor splice variants that underlie any given AMPA receptor-mediated response (Sekiguchi *et al*., 1998; Shen & Yang, 1999; Shen *et al*., 1999a, b). Hence, we undertook an in-depth analysis of the kinetics of PEPA action on AMPA receptors.

For our purposes, outside-out patch clamp recordings were performed using *Xenopus* oocytes expressing recombinant rat AMPA receptors. We investigated the effects of PEPA on deactivation, desensitization, and recovery from desensitization of glutamate responses mediated by the expressed AMPA receptors. To further explore the molecular and kinetic mechanisms involved, we carried out simulations of the effects of PEPA using an AMPA receptor kinetic model. We found that PEPA, aniracetam, and cyclothiazide differentially modulate AMPA receptor kinetics, and that attenuation of the extent of desensitization is the principal mechanism underlying potentiation by PEPA. Our simulation studies suggest that different (but overlapping) mechanisms govern the actions of PEPA on GluR3-flip and GluR3-flop.

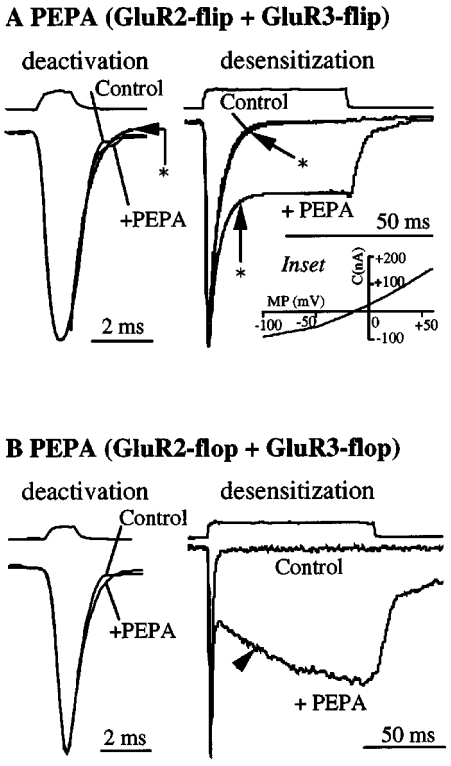


Figure 1 Effect of PEPA on desensitization and deactivation of recombinant AMPA receptors. The effects of 100 μM PEPA on the response of AMPA receptors to 10 mM glutamate pulses (1 ms for deactivation, and 50 or 100 ms for desensitization) in outside-out patches excised from oocytes expressing AMPA receptors. In each record in A and B, the peak amplitude of the response with PEPA (+PEPA) was scaled to that in the absence of PEPA (Control). Each record represents the average of 3–10 responses. In this and subsequent figures, the trace above each record represents the open tip junction currents which indicate the solution exchange in each experiment. The arrowhead in B indicates the slow onset phase of PEPA's action. The traces labelled by * (superimposed to actual responses) are single exponential fits (a trace in 'deactivation' is for+PEPA). *Inset* to A: an example of the I–V relationship recorded by two-electrode voltage clamp recordings from an oocyte injected with GluR2-flip and GluR3-flip cRNAs (4:1 by weight).

Methods

Outside-out recordings

Expression of AMPA receptors in *Xenopus* oocytes was performed as previously described (Sekiguchi *et al*., 1997). Briefly, cRNA was transcribed *in vitro* from cDNA encoding rat AMPA receptors. Oocytes were defolliculated by treatment with collagenase (Type I, Sigma, St. Louis, MO, U.S.A.), and injected with the cRNA. After incubation at 20°C for 3–7 days, the expression of AMPA receptors in oocytes was initially analysed by conventional two-electrode voltage-clamp recordings as previously described (Sekiguchi *et al*., 1997). Oocytes that exhibited robust response to glutamate were used in outside-out recordings. When GluR2 was expressed, the current-voltage (I–V) relationship was measured by two-electrode voltage-clamp recordings to determine the contribution of GluR2-containing receptors in the glutamate response. Membrane potential was ramped from −100 to +60 mV before and during application of glutamate, and the I–V relationship was calculated. The majority of oocytes that were injected with a mixture of cRNAs encoding GluR2 and GluR3 (4:1 by weight) exhibited outward rectification during the glutamate response (See Figure 1A-*inset*), and such oocytes were used in outside-out recordings.

Selected oocytes were incubated at room temperature in frog Ringer's solution (see recipe below) in which all salt concentrations except $CaCl_2$ were doubled. After 10–30 min, the vitelline membrane surrounding the oocytes was removed manually and the oocytes were used for outside-out recording. Outside-out patch recording was carried out using an EPC-9 amplifier (HEKA, Lambrecht, Germany) under continuous perfusion with frog Ringer's solution (115 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 10 mM HEPES, adjusted to pH 7.2 with NaOH). The patch pipette was prepared from borosilicate glass capillaries (WPI, Sarasota, FL, U.S.A.) and had 3–5 MΩ input resistance when filled with 100 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with KOH. Responses were filtered at 10 KHz and digitized at 20–50 μs/point. The holding potential was maintained at −60 mV. Fast application of glutamate was performed using the methods described by Jonas (1995). Briefly, glutamate was applied by perfusion of the patch membrane with a θ tube driven by a piezo manipulator (PZ-150M, Burleigh, Fishers, NY, U.S.A.). One end of the θ tube was processed to 100 μm in diameter at the barrel. The other end of each barrel was connected to a silicon tube, and each silicon tube was branched into two tubes that were connected to separate solution reservoirs. Therefore, four reservoirs were prepared for a θ tube. Individual reservoirs contained

frog Ringer's solution (control), or frog Ringer's solution containing either 10 mM glutamate, 100 μM PEPA, or 10 mM glutamate + 100 μM PEPA. The two barrels of the θ tube were first filled with frog Ringer's solution and the solution containing 10 mM glutamate, respectively. To record control glutamate responses, a patch membrane was placed into the stream of control solution from one barrel, and the θ tube was moved by the piezo manipulator so that a patch membrane was then perfused with 10 mM glutamate-containing solution from the other barrel. After recording, the solutions in the θ tube were replaced by frog Ringer's solution containing 100 μM PEPA in one barrel and by the same solution containing 100 μM PEPA + 10 mM glutamate in the other barrel. Approximately 90 s were required to replace the solutions in the θ tube (investigated using bromophenol blue), and glutamate pulses in the presence of PEPA were initiated 120 s after the start of solution replacement. Thus, for PEPA experiments, AMPA receptors in each patch membrane were exposed to 100 μM PEPA for 30 s at the start of recording. Since it takes only a few seconds to achieve maximal PEPA action in the AMPA receptors expressed in human embryonic kidney 293 cells (Sekiguchi *et al*., 1997), it is likely that AMPA receptors in our patch experiments became equilibrated with PEPA by the start of the recording.

After recording, the patch membrane was blown off, and the junction current between the control solution and 10% frog Ringer's solution was measured to monitor solution exchange without moving the patch pipette and the θ tube. Responses to glutamate having a 20–80% rise time less than 500 μs were used for analysis.

Effects of PEPA upon amplitude of the response for 1 ms glutamate pulse were expressed as (current amplitude of response during application of 100 μM PEPA)/ (amplitude of the response before application of PEPA) $\times$ 100% (I_{pepa}/I_{cont}).

The decay phase of the response was fit to single exponential functions using Igor Pro (WaveMetrics, Lake Oswego, OR, U.S.A.) or PulseFit (HEKA). The decay time constant for deactivation (τ_{dea}) or desensitization (τ_{des}) was calculated from the fitted curve, and τ was the response decay time required to attain e^{-1} (approximately 36.8%) of the maximal amplitude, as determined from the fitted curve. The start points for curve fitting ranged from 65–95% of the maximal amplitude of actual responses. The extent of desensitization was expressed as current amplitude of the steady-state response/current amplitude of the initial peak-like response (I_{ss}/I_{peak}). The amplitude of steady-state currents was measured at 50 ms from the start of glutamate application in the case of GluR1-flip, GluR1-flop, GluR3-flip and GluR2-flip + GluR3-flip, at 100 ms for GluR2-flop + GluR3-flop, and at 150 ms for GluR3-flop. Differences in measurement time were necessary to allow for the slow onset of PEPA action in the latter two cases (see Figures 1B, 6B-desensitization). Each onset phase of the response in the presence of PEPA in GluR3-flop (indicated by an arrowhead in Figure 6B-desensitization) was fit to a single exponential function, and the time constant for potentiation (τ_{pot}) was calculated from the fitted curve (τ_{pot} = the time given e^{-1} potentiation)

Recovery from desensitization was measured by plotting the ratio of the second response to the first response (I_{2nd}/I_{1st},%) as a function of time. This plot was fitted to a single exponential function such that the maximal suppression in the response to the second glutamate pulse was obtained at a 5-ms interpulse interval. The exception was GluR1-flip for which the maximum suppression was obtained at 10 ms (the amplitude of the response for the second pulse was smaller with an interpulse interval of 10 ms compared to 5 ms). The time constant of recovery (τ_{rec}) indicated the time (interpulse interval) when the suppression became e^{-1} of the maximal suppression.

Simulation

Kinetic simulation studies were conducted using the computer program FastFlow (Benveniste *et al*., 1990). Initial experimental conditions were as follows: holding potential, −60 mV; number of receptors, 100; conductance of open channels; 10 pS. The initial rate constants were taken from the model by Raman & Trussell (1995) for AMPA receptors in the nucleus magnocellularis and the model by Partin *et al*. (1996) for GluR1 and were modified to reproduce the experimental responses of recombinant GluR3 in oocytes. We also utilized rate constants of Arai *et al*. (1996a) used in simulation of the action of BDP-20 on AMPA receptors in neurons in CA1 and CA3 fields of rat hippocampus. The rate constants were set to satisfy microscopic reversibility in the cycle.

Drugs

PEPA was supplied by Nihonsuisan Co. Ltd. (Hachioji, Japan). Glutamate and aniracetam were purchased from Sigma. Cyclothiazide was from RBI (Natick, MA, U.S.A.).

Statistics

For comparison of two data groups, the two-tailed Student's *t*-test was used.

Results

Effects of PEPA on AMPA receptor kinetics

Figure 1A,B show the effects of 100 μM PEPA on the response of AMPA receptors to 10 mM glutamate pulses (1 ms for deactivation, and 50 or 100 ms for desensitization) in outside-out patches excised from oocytes expressing AMPA receptors denoted. In each record, the peak amplitude of the response to PEPA was scaled to that in its absence. PEPA did not significantly affect responses in 1-ms pulses of glutamate in either GluR2-flip + GluR3-flip (Figure 1A) or GluR2-flop + GluR3-flop (Figure 1B). For GluR2-flip + GluR3-flip, τ_{dea} values were 541 ± 77 (glutamate alone, n = 5) and 569 ± 25 μs (glutamate + PEPA, n = 5); and for GluR2-flop + GluR3-flop the values were 647 ± 39 (n = 6) and 681 ± 61 μs (n = 6), respectively. In contrast, PEPA suppressed the extent of desensitization (desensitization in Figure 1A,B). For GluR2-flip + GluR3-flip, I_{ss}/I_{peak} values were 4.2 ± 2.0% (glutamate alone, n = 5) and 31.8 ± 1.3% (glutamate + PEPA, n = 5); and for GluR2-flop + GluR3-flop the values were 0.7 ± 0.2% (n = 6) and 55.9 ± 9.1% (n = 6), respectively. The effects of PEPA on desensitization of

GluR2-flop + GluR3-flop and GluR3-flop (see Figure 6) subunits were unique, in that the onset of drug action was very slow (see arrowhead in Figure 1B for GluR2-flop + GluR3-flop). This phenomenon was observed consistently in experiments using GluR2-flop + GluR3-flop or GluR3-flop alone. As we mentioned in detail in Methods section, it is likely that AMPA receptors in our patch experiments became equilibrated with PEPA at the glutamate application. PEPA did not slow the onset of desensitization in both GluR2-flip + GluR3-flip and GluR2-flop + GluR3-flop (Figure 1A,B). The τ_{des} values were 3.8 ± 0.4 (glutamate alone, $n = 5$) and 3.8 ± 0.3 ms (glutamate + PEPA, $n = 5$) for the flip combination, and the values were 1.1 ± 0.1 ($n = 6$) and 1.0 ± 0.2 ms ($n = 6$) for the flop combination. Figure 1A-(*inset*) shows an I–V relationship measured by two-electrode voltage clamp recordings from an oocyte injected with GluR2-flip and GluR3-flip (see Methods). It is well known that AMPA receptors containing GluR2 show outward rectification while GluR2-less receptors show inward rectification (Verdoorn *et al*., 1991). The fact that this I–V relationship is outwardly rectifying implies that current responses in the oocytes used for outside-out recordings were mediated through GluR2-containing receptors.

Experiments similar to those in Figure 1 were performed for GluR1-flip, GluR1-flop, and GluR3-flip and GluR3-flop, and τ_{dea}, τ_{des}, and I_{ss}/I_{peak} were calculated from the records (Figure 2). No significant change in τ_{dea} was evident in GluRs other than GluR3-flop in which a very slight (1.3 fold) but significant ($P = 0.0293$) increase in τ_{dea} was observed (Figure 2A, see Figure 6 also). This slight effect was not observed in oocytes co-expressing GluR2-flop and GluR3-flop (Figures 1 and 2A). The τ_{des} values in the ($\pm$) PEPA experiments were not outstandingly different in all AMPA receptor combinations tested (Figure 2B). The value of I_{ss}/I_{peak} was increased by PEPA in all the AMPA receptor combinations tested (Figure 2C). The fold increase of the I_{ss}/I_{peak} value by PEPA is shown in Figure 2D, and the rank order is GluR3-flop > GluR2-flop + GluR3-flop > GluR3-flip > GluR2-flip + GluR3-flip > GluR1-flop > GluR1-flip. The increase of I_{ss}/I_{peak} was more pronounced in flop variants than in flip variants for a given subunit (combination), and the increase was also more pronounced for GluR3 than for GluR1 subunits. This flop- and GluR3-preference is consistent with our previous studies using slower glutamate application (Sekiguchi *et al*., 1997).

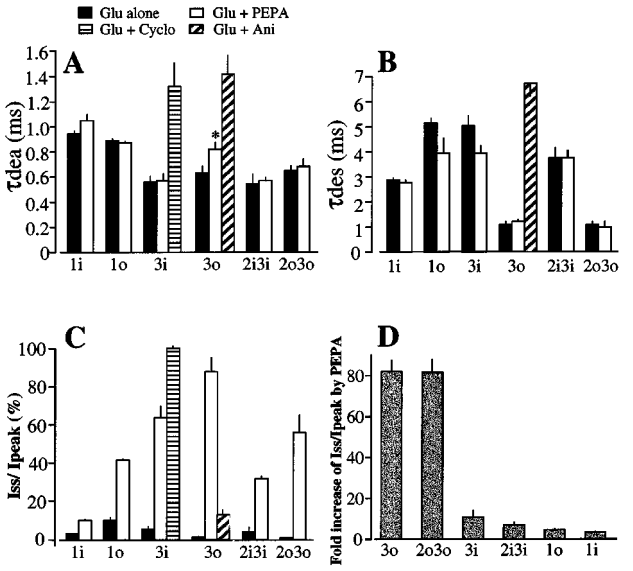


Figure 2 Effect of PEPA on the deactivation time constant (τ_{dea}, A), the desensitization time constant (τ_{des}, B), and extent of desensitization (I_{ss}/I_{peak}, C), and fold increase in the I_{ss}/I_{peak} by PEPA (D), in recombinant AMPA receptors. The response of AMPA receptors to 10 mM glutamate pulses, 1 ms for deactivation, and 50, 100 (for GluR2-flop + GluR3-flop), or 150 (for GluR3-flop) ms for desensitization, was recorded with or without 100 μM PEPA in outside-out patches excised from oocytes expressing AMPA receptors (i: flip, o: flop). The τ_{dea}, τ_{des}, and I_{ss}/I_{peak} values were calculated from the records and expressed as mean $\pm$ standard error of mean (s.e.m., $n = 4-7$ patches). Fold increase in $I_{ss}/I_{peak} = (I_{ss}/I_{peak}$ in the presence of PEPA)/(I_{ss}/I_{peak} of the control glutamate response). The amplitude of the control glutamate responses ranged from 14 to 200 pA. The amplitude in the presence of PEPA was 87 ± 8 ($n = 4$), 94 ± 8 ($n = 5$), 103 ± 7 ($n = 5$), 80 ± 7 ($n = 7$), 100 ± 10 ($n = 5$), and 86 ± 7 ($n = 6$)% of the control responses (I_{pepa}/I_{cont}) in GluR1-flip, GluR1-flop, GluR3-flip, GluR3-flop, GluR2-flip + GluR3-flip and GluR2-flop + GluR3-flop, respectively. Similar data in the presence of cyclothiazide (100 μM, $n = 4$) and aniracetam (3 mM, $n = 7$) were included for comparison. *$P = 0.0293$ when compared with values for glutamate alone.

Comparison of PEPA with aniracetam and cyclothiazide

We analysed the effect of aniracetam and cyclothiazide on AMPA receptor kinetics as previously reported (Partin *et al*., 1996; Arai *et al*., 1996a) for direct comparison with our results for PEPA. GluR3-flop was chosen for aniracetam experiments since it shows a weak preference for GluR3-flop over GluR3-flip (Sekiguchi *et al*., 1997), and GluR3-flip was chosen for cyclothiazide experiments since it shows preference for flip variants (Partin *et al*., 1994). Aniracetam (3 mM) slows the deactivation of glutamate responses in GluR3-flop (Figure 3A), and likewise for cyclothiazide (100 μM) and GluR3-flip (Figure 3B); corresponding τ_{dea} were 1430 ± 178 ($n = 5$, $\sim$2.3 fold increase over glutamate alone, 629 μs, see Figure 6D) and 1315 ± 213 μs (n = 4, $\sim$2.4 fold increase over glutamate alone, 558 μs, see Figure 5D), respectively. The τ_{dea} for aniracetam was $\sim$1.7 fold greater for PEPA for GluR3-flop (1430 *vs* 818, see Figure 6C), and PEPA did not significantly affect τ_{dea} for GluR3-flip (Figure 2). These results suggest that PEPA modulates GluR3 deactivation less effectively than aniracetam or cyclothiazide.

Aniracetam modulates both the onset and extent of desensitization in GluR3-flop (Figure 3A, desensitization). Namely, the I_{ss}/I_{peak} value in the presence of aniracetam is $13.5 \pm 3.3\%$ (n = 5), an $\sim$12.3 fold increase over that with glutamate alone (1.1%, see Figure 6D). The τ_{des} value also increased from 1.1 ± 0.1 (glutamate alone; see Figure 6C) to 6.7 ± 0.5 ms ($n = 5$). Cyclothiazide abolished desensitization in GluR3-flip (Figure 3B, desensitization). These results suggest that PEPA, aniracetam and cyclothiazide differentially modulate AMPA receptor desensitization. Figure 2A–C compares kinetic parameters of AMPA receptors in the presence of PEPA *vs* aniracetam or cyclothiazide.

Recovery from desensitization

Arai and Lynch (1998) reported that some AMPA receptor potentiators greatly facilitate receptor recovery from desensi-

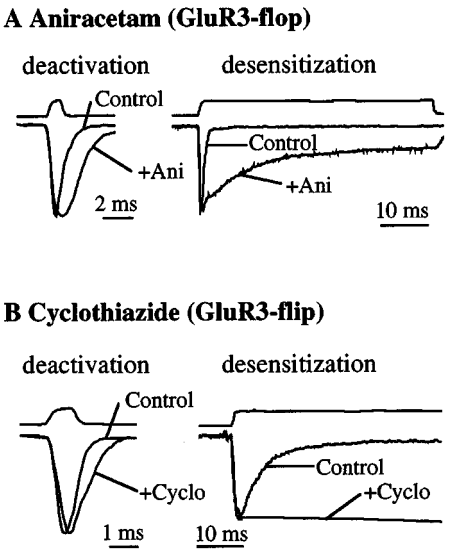


Figure 3 Effect of cyclothiazide and aniracetam on desensitization and deactivation of recombinant AMPA receptors. The effects of 3 mM aniracetam (A) and 100 μM cyclothiazide (B) on the response to 10 mM glutamate pulses (1 ms for deactivation, and 50 ms for desensitization) in outside-out patches excised from oocytes expressing GluR3-flip and GluR3-flop, respectively. In each record in A and B, the peak amplitude of the response with potentiators (+Cyclo or +Ani) was scaled to that in the absence of potentiators (Control). Each record represents the average of 3–10 responses.

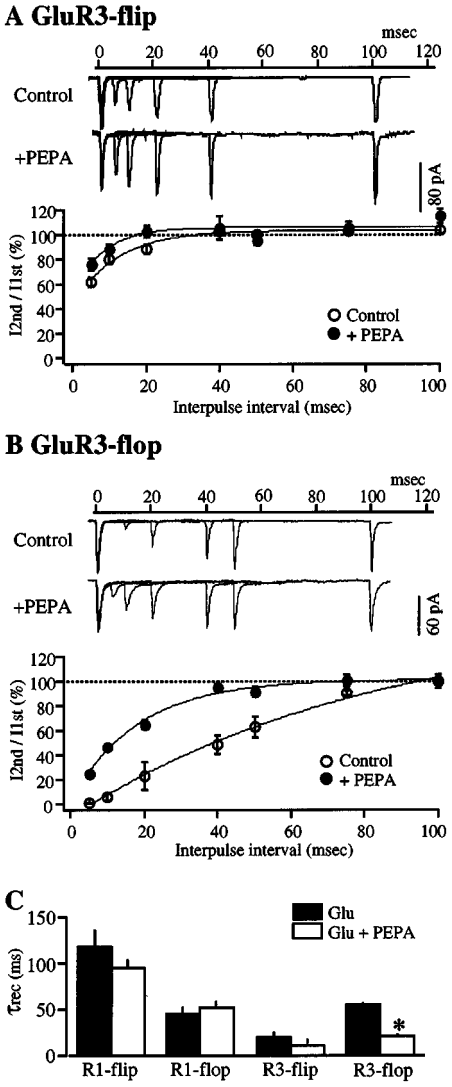


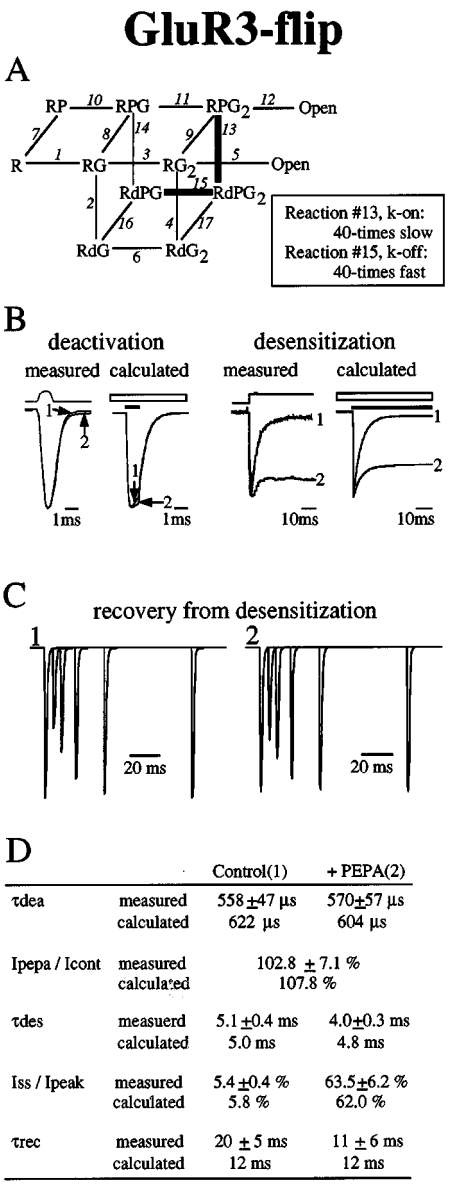
Figure 4 Twin pulse application of 1 ms glutamate pulses. Two 1-ms glutamate pulses were applied (over varying interpulse intervals) to a membrane patch excised from an oocyte expressing GluR3-flip (A) or GluR3-flop (B), with or without PEPA (100 μM). The records were superimposed and scaled to the amplitude of the first response. The graphs below the superimposed records show the change in the amplitude of the second response relative to the first response as a function of the interpulse interval. The values are mean $\pm$ s.e.m. from 4–5 experiments. The τ_{rec} values were calculated for GluR1-flip, GluR1-flop, GluR3-flip, and GluR3-flop with and without PEPA, and are compared in (C). *$P<0.0001$.

tization. We examined the effects of PEPA on recovery from desensitization by applying two 1-ms glutamate pulses to outside-out membrane patches using interpulse intervals of 5, 10, 20, 40, 50 (for GluR3-flop only), and 100 ms. Data for the superimposed records are presented in Figure 4A (GluR3-flip, 5 records) and Figure 4B (GluR3-flop, 6 records). The graph below each record shows the mean $\pm$ standard error of mean ($n=4-5$) of I_{2nd}/I_{1st}. The amplitude of the response to the second glutamate pulse was smaller than that of the first if the interpulse interval was sufficiently short, but with a longer interval the amplitude of the response recovered to a level equal to the first response. The suppression was much more potent in GluR3-flop than in GluR3-flip, and recovery was faster in GluR3-flip than in GluR3-flop (Figure 4). PEPA accelerated recovery in GluR3-flop (Figure 4B) but not in GluR3-flip (Figure 4A). Figure 4C shows the calculated recovery time constants (τ_{rec}). The τ_{rec} was significantly reduced ($P<0.0001$) by $\sim$2.6 fold for GluR3-flop in the presence of PEPA (55 $\pm$ 2 ms for glutamate alone *vs* 21 $\pm$ 2 ms for glutamate + PEPA, $n=6$ for each, Figure 4C), but was not significantly changed for GluR3-flip (20 $\pm$ 5 and 11 $\pm$ 6 ms for glutamate alone and glutamate + PEPA, respectively, $n=4$). PEPA did not accelerate recovery in GluR1-flip or GluR1-flop (Figure 4C).

Simulation study for PEPA action on GluR3-flip

To investigate differences in the molecular mechanism by which PEPA affects AMPA receptors either containing or missing GluR3-flop subunits, simulation studies were performed based on an AMPA receptor kinetic model (Partin *et al*., 1996). This model describes binding of two glutamate molecules for one receptor and is utilized to simulate actions of aniracetam and cyclothiazide upon GluR1 (Partin *et al*., 1996). Figure 5A shows a 12-state kinetic model used to simulate the action of PEPA on GluR3-flip. In this and subsequent models, we hypothesized that PEPA does not change single-channel conductance. Also, we simplified kinetics of AMPA receptor, for example, native AMPA receptors are known to have sub-conductance states (Howe *et al*., 1991), but only one conductance state was set in the model.

First, the parameters for the effect of glutamate (10 mM) alone on GluR3-flip were set such that the simulated response mimicked the actual response (trace 1 of 'measured' in Figure 5B). The parameter set employed is listed in Table 1. The 'calculated' traces (trace 1) for both deactivation and



D

		Control(1)	+ PEPA(2)
τdea	measured	558±47 μs	570±57 μs
	calculated	622 μs	604 μs
Ipepa / Icont	measured	102.8 ± 7.1 %	
	calculated	107.8 %	
τdes	measuerd	5.1±0.4 ms	4.0±0.3 ms
	calculated	5.0 ms	4.8 ms
Iss / Ipeak	measured	5.4±0.4 %	63.5±6.2 %
	calculated	5.8 %	62.0 %
τrec	measured	20 ± 5 ms	11 ± 6 ms
	calculated	12 ms	12 ms

Figure 5 Simulation of PEPA's action on GluR3-flip. (A) The kinetic model used for simulation. R = sensitized receptor, Rd = desensitized receptor, G = glutamate, P = PEPA, Open = open state. The numbers 1 to 17 denote those reactions referred to in the text. Bold lines indicate reactions that were changed for the purpose of simulating the action of PEPA (denoted in the box). (B) The traces below 'measured' are actual responses from outside-out patches excised from oocytes expressing GluR3-flip, and the traces below 'calculated' are simulated responses obtained using the rate constants listed in Table 1. The traces labelled '1' are for glutamate alone (10 mM), and those labelled '2' are for glutamate (10 mM) + PEPA (100 μM). The open and closed bars for 'calculated', respectively, show the periods of PEPA and glutamate application. Deactivation and desensitization were measured with 1 and 50 ms glutamate pulses, respectively. (C) The simulated responses for twin glutamate pulses (1 ms) with various interpulse intervals (5, 10, 20, 40 and 100 ms) are superimposed. 1: control, 2: + PEPA (the parameter set in Table 1 was used). (D) The values of τ_{dea}, I_{pepa}/I_{cont}, τ_{des}, I_{ss}/I_{peak}, and τ_{rec} calculated in actual (= measured) and simulated (= calculated) responses for both Control (from no. 1 traces) and + PEPA (from no. 2 traces).

desensitization in Figure 5B are simulated responses to glutamate alone. The τ_{dea}, τ_{des}, and I_{ss}/I_{peak} values of the simulated response were close to those of the actual response, as shown in Figure 5D – 'Control (1)'.

Next, a parameter set for simulation of the effect of PEPA on deactivation, desensitization, and recovery from desensitization was established (Table 1). The 'calculated' traces (trace 2) in Figure 5B are the simulated responses in which the parameters of reaction no. 13 and no. 15 of the kinetic model (bold lines in the model of Figure 5A) were altered (as listed in Figure 5A). Namely, the transition from RPG_2 to $RdPG_2$ (desensitization of glutamate- and PEPA-bound receptor) was slowed 40 fold *vs* the control (glutamate alone, reaction no.4), and the transition from $RdPG_2$ to RdPG (dissociation of one glutamate from a desensitized receptor) was accelerated 40 fold *vs* the control (reaction no.6, see Discussion). Under these conditions, the values of I_{pepa}/I_{cont}, τ_{dea}, τ_{des}, and I_{ss}/I_{peak} of the simulated response were close to those of the actual response (see each trace 2 of 'measured'), as listed in Figure 5D – '+PEPA (2)'. Figure 5C shows the recovery from desensitization without (trace 1) and with (trace 2) PEPA, which is simulated using the parameter set used above. PEPA did not affect recovery from desensitization, similar to what was observed in the actual responses (Figure 4A). The τ_{rec} values of the simulated response were close to those values of the actual responses (Figure 5D).

Simulation study for PEPA action on GluR3-flop

Figure 6A shows a 13-state kinetic model in which the 13th state represents a PEPA-bound receptor to which glutamate cannot bind (RP*). This model was used to simulate the action of PEPA on GluR3-flop. There are two principal rationales for adding the 13th state to the previous 12-state model. First, in order to achieve slow onset of drug action, the binding of glutamate to a PEPA-bound receptor (RP) must necessarily be inhibited in the initial period of the reaction. This can be achieved by invoking the existence of a PEPA-bound receptor to which glutamate cannot bind, namely RP*, which would attain equilibrium with the PEPA-bound receptor. Second, the entirety of the slow-onset phase of PEPA's action on GluR3-flop desensitization (arrowhead in 'measured' of desensitization of Figure 6B) fits a single exponential function. The fitted curve (*) can be precisely superimposed on the actual record (trace 2 of 'measured' of desensitization in Figure 6B). Thus, this measured data can be modelled simply by adding the 13th state (equilibration between two PEPA-bound states) to the 12-state model.

Initially, parameters were set to simulate responses to glutamate alone in GluR3-flop. This was achieved by changing mainly the time constants of reactions no. 4 and no. 6 (R3-flop of Table 1) on the basis of those values determined for GluR3-flip. Namely, in reaction no. 4, desensitization of RG_2 was accelerated (approximately 12 fold) and re-sensitization of RdG_2 was slowed (8 fold), and in reaction no. 6, the dissociation of glutamate from RdG2 was slowed (about 30 fold). Trace 1 of 'measured' in Figure 6B shows the actual responses. Trace 1 of 'calculated' in Figure 6B is the simulated response by glutamate alone prepared using the parameter set in Table 1. The τ_{dea}, τ_{des}, and I_{ss}/I_{peak} values of the simulated response were close to those of the actual response by glutamate alone, as listed in Figure 6D – 'Control (1)'.

Table 1 Rate constants used for the simulation of the action of PEPA

		R3-flip		*R3-flop*	
Reaction		$k(+)\ s^{-1}$	$k(-)\ s^{-1}$	$k(+)\ s^{-1}$	$k(-)\ s^{-1}$
1	R–RG	$2\times10^7\ M^{-1}$	300	$2\times10^7\ M^{-1}$	300
2	RG–RdG	1000	500	2000	300
3	RG–RG_2	$10^7\ M^{-1}$	100,000	$10^7\ M^{-1}$	100,000
4	RG_2–RdG_2	1300	6	16,000	0.75
5	RG_2–Open	17,500	2000	15,000	1800
6	RdG–RdG_2	$10^7\ M^{-1}$	923	$10^7\ M^{-1}$	31.25
7	R–RP	$10^5\ M^{-1}$	0.1	$10^5\ M^{-1}$	0.1
8	RG–RPG	$10^5\ M^{-1}$	0.1	$10^5\ M^{-1}$	0.025
9	RG_2–RPG_2	$10^5\ M^{-1}$	0.1	$10^5\ M^{-1}$	0.0025
10	RP–RPG	$2\times10^7\ M^{-1}$	300	$2\times10^7\ M^{-1}$	**75**
11	RPG–RPG_2	$10^7\ M^{-1}$	100,000	$10^7\ M^{-1}$	**10,000**
12	RPG_2–Open	17,500	2000	**30,000**	**3600**
13	RPG_2–$RdPG_2$	**32.5**	6	**8000**	**525**
14	RPG–RdPG	1000	500	2000	**600**
15	RdPG–$RdPG_2$	$10^7\ M^{-1}$	**36,923**	$10^7\ M^{-1}$	**2187.5**
16	RdG–RdPG	$10^5\ M^{-1}$	0.1	$10^5\ M^{-1}$	0.05
17	RdG_2–$RdPG_2$	$10^5\ M^{-1}$	4	$10^5\ M^{-1}$	3.5
18	RP–RP*	–	–	**15**	**12.5**

The bold values were set to simulate the glutamate response in the presence of PEPA. The k(+) is the rate constant from the left state to the right state and the k(−) is the rate constant from the right state to the left state.

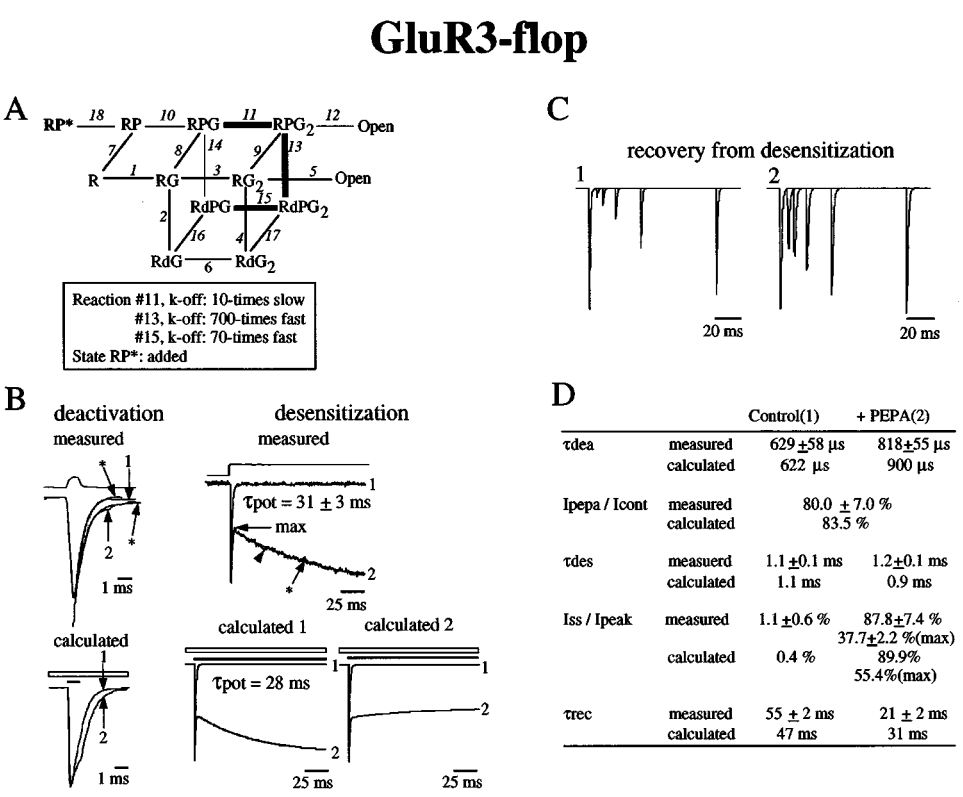


Figure 6 Simulation of PEPA's action on GluR3-flop. (A) The kinetic model used for simulation. R = sensitized receptor, Rd = desensitized receptor, G = glutamate, P = PEPA, Open = open state, RP* = PEPA-bound receptor to which glutamate cannot bind. Numbers 1 to 18 denote those reactions referred to in the text. Bold lines indicate major reactions (>10 fold change in the parameter, denoted in the box) that were changed for the purpose of simulating the action of PEPA. (B) The traces below 'measured' are actual responses from outside-out patches excised from oocytes expressing GluR3-flop, and the traces below 'calculated' or 'calculated 1' are simulated responses obtained using the rate constants listed in Table 1. 'Calculated 2' represents the simulated responses when state RP* is removed from the parameter set in Table 1. The traces labelled as 1 are for glutamate alone (10 mM), those labelled as 2 are for glutamate (10 mM) + PEPA (100 μM), and those labelled by * are single exponential fits. The open and closed bars for 'calculated' show the periods of PEPA and glutamate application, respectively. Deactivation and desensitization were measured with 1 and 150 ms glutamate pulses, respectively. In 'measured' under desensitization, an arrowhead indicates 'slow' onset phase, and an arrow indicates the point of maximal desensitization (max). (C) The simulated responses for twin glutamate pulses (1 ms) with various interpulse intervals (5, 10, 20, 40 and 100 ms) were superimposed. 1: control, 2: + PEPA (parameter set in Table 1). (D) The values of τ_{dea}, I_{pepa}/I_{cont}, τ_{des}, I_{ss}/I_{peak}, and τ_{rec} calculated in actual (= measured) and simulated (= calculated) responses for both Control (from no. 1 traces) and + PEPA (from no. 2 traces). For I_{ss}/I_{peak}, the values with '(max)' were calculated at the point of maximal desensitization (See Figure 6B-desensitization-'measured').

Trace 2 of 'calculated' in Figure 6B shows the stimulated responses in which the parameters of reactions listed in Table 1 were altered as denoted, to simulate actions of PEPA on GluR3-flop. The reactions with more than a 10 fold alteration in the parameters are no. 11, no. 13, and no. 15 (indicated by bold lines in the model). Namely, the dissociation of glutamate from RPG_2 (k-off in reaction no. 11) was slowed 10 fold *vs* the control (reaction no. 3), the resensitization of $RdPG_2$ (k-off in reaction no. 13) was accelerated 700 fold *vs* the control (reaction no. 4), and the dissociation of glutamate from $RdPG_2$ (k-off in reaction no. 15) was accelerated 70 fold *vs* the control (reaction no. 6). Slowing of k-off in reaction no. 11 was in reference to our previous result that PEPA increased the apparent affinity of GluR3-flop for glutamate approximately 7 fold (Sekiguchi *et al*., 1997). Under these conditions, the values of I_{pepa}/I_{cont}, τ_{dea}, τ_{des}, and I_{ss}/I_{peak} of the simulated response were relatively close to those of the actual response with PEPA (trace 2 of 'measured'), as listed in Figure 6D–'+PEPA (2)'. In particular, this simulation achieved 'the slow onset of action of PEPA' on desensitization (trace 2 of desensitization-'calculated 1' of Figure 6B), and the calculated τ_{pot} (28 ms) was close to the value for the actual response (31 ± 3 ms, $n = 5$). If the 13th state, RP*, is removed from the model and the parameters are changed as listed in Table 1, simulation gives the response suggested in 'calculated 2' of desensitization (Figure 6B), which cannot achieve a slow onset of PEPA action.

Figure 6C–'recovery from desensitization' shows the recovery from desensitization without (trace 1) and with (trace 2) PEPA, which is simulated using the parameter set used above. PEPA accelerated the recovery from desensitization as shown by the actual responses (Figure 4A). The τ_{rec} values of the simulated responses were close, although not identical, to those of the actual responses (Figure 6D).

Discussion

Our data suggest that (1) PEPA does not influence AMPA receptor deactivation, with the exception of a slight slowing (1.3 fold) of deactivation in homomeric GluR3-flop receptors, (2) PEPA modulates the extent, but not the onset of desensitization, and (3) PEPA accelerates recovery from desensitization in GluR3-flop-containing AMPA receptors only. On the basis of these data, we carried out kinetic simulation studies.

We previously reported that PEPA preferentially affects AMPA receptor flop-splice variants over flip variants, and that PEPA is about 100 fold more potent than aniracetam with respect to flop-variants (Sekiguchi *et al*., 1997). Here, a detailed examination of the effects of PEPA on AMPA receptor kinetics was undertaken using a glutamate application system that is faster than that used in our previous study (Sekiguchi *et al*., 1997). Our results suggest that the principal mechanism by which PEPA potentiates AMPA receptors is by attenuating the extent of receptor desensitization. This attenuation is more potent in flop-splice variants than in the flip variants of a given AMPA receptor, and more potent in receptors containing GluR3 than GluR1. These variant/subunit preferences are in agreement with the results from two-electrode voltage clamp recordings in *Xenopus* oocytes and whole cell patch clamp recordings in culture cells (Sekiguchi *et al*., 1997). The consistency of the data supports our conclusion that attenuation of the extent of desensitization is a central mechanism by which PEPA potentiates AMPA receptor activity. In other words, the responses obtained in our oocyte system (Sekiguchi *et al*., 1997) correspond predominantly to steady-state currents recorded in the outside-out patch system used in the present study.

PEPA, aniracetam, and cyclothiazide differentially modulate AMPA receptor kinetics. Aniracetam modulates deactivation in GluR3-flop (2.3 fold; present study) as well as in GluR1-flip and GluR1-flop (Partin *et al*., 1996). PEPA does not affect desensitization in GluR1-flip and GluR1-flop, and modulation of deactivation by PEPA in GluR3-flop in our study was weaker than that observed using aniracetam. Cyclothiazide modulates deactivation in GluR3-flip (present study) and GluR1-flip (Partin *et al*., 1996), but does not affect deactivation in GluR1-flop (Partin *et al*., 1996). PEPA does not affect deactivation in GluR1-flip, GluR1-flop, or GluR3-flip. These results suggest that PEPA affects deactivation to a much lesser extent than aniracetam or cyclothiazide.

With respect to desensitization, aniracetam modulates both the onset and extent of desensitization in GluR3-flop (present study) as well as in GluR1-flop (Partin *et al*., 1996). Cyclothiazide abolishes desensitization in GluR3-flip (present study) and GluR1-flip (Partin *et al*., 1996). In contrast, PEPA attenuates the extent of desensitization without slowing the onset. Thus, while all three of these AMPA potentiators affect desensitization, the mechanisms by which they exert their effects are not uniform.

The actions of PEPA upon GluR3-flop were mimicked on the hypothesis that there is a 13th state, PEPA-bound receptor to which glutamate cannot bind (RP*), in the actions. The RP* was not necessary for the actions of PEPA upon GluR3-flip. In the 13-state model, the initial peak-like response of GluR3-flop to glutamate would be suppressed in presence of PEPA because we assumed that RP* is a non-conducting state. Indeed, the simulated I_{pepa}/I_{cont} is 83.5%, which is very close to the measured value of $80.0 \pm 7.0\%$. Thus, the 13-state model can explain the effects of PEPA upon the amplitude of the initial peal-like response, as well as the slow onset. However, it is important to note that the simulation studies do not allow unambiguous separation of effects on desensitization, binding and gating. Studies such as the single channel measurement will be necessary for further exploring the differences in PEPA's actions between GluR3-flop and GluR3-flip.

In conclusion, we investigated the effects of PEPA on the receptor kinetics using outside-out patch clamp recordings as well as kinetic simulations. Our results indicate that PEPA potentiates AMPA receptors principally by attenuating the extent of receptor desensitization. Also, PEPA is much more selective for desensitization over deactivation when compared with aniracetam and cyclothiazide. PEPA differentially affects GluR3-flip and GluR3-flop, and these differences were explored by kinetic simulation studies of AMPA receptor. To simulate the slow onset of PEPA's action on GluR3-flop, it is necessary to invoke a PEPA-bound receptor to which glutamate cannot bind, termed RP*. These results are informative with regard to the use of PEPA as a pharmacological tool in academic studies, and will also be helpful for the development of AMPA receptor potentiators for clinical purposes.

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