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Auxiliary proteins promote modal gating of AMPA- and kainate-type glutamate receptors

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Abstract

The gating behavior of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors is modulated by association with the auxiliary proteins: transmembrane AMPA receptor regulatory proteins (TARPs) and neuropilin tolloid-like (Netos), respectively. Although the mechanisms underlying receptor modulation differ for both AMPA and kainate receptors, association with these auxiliary subunits results in the appearance of a slow component in the decay of ensemble responses to rapid applications of saturating concentrations of glutamate. We show here that these components arise from distinct gating behaviors, characterized by substantially higher open probability (P_{open}), which we only observe when core subunits are associated with their respective auxiliary partners. We refer to these behaviors as gating modes, because individual receptors switch between the low- and high- P_{open} gating on a time-scale of seconds. At any given time, association of AMPA and kainate receptors with their auxiliary subunits results in a heterogeneous receptor population, some of which are in the high- P_{open} mode and others that display gating behavior similar to that seen for receptors formed from core subunits alone. While the switching between modes is infrequent, the presence of receptors displaying both types of gating has a large impact on both the kinetics and amplitude of ensemble currents similar to those seen at synapses.

Keywords

glutamate; kinetics; modulation; Neto2; TARPs

Introduction

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors are glutamate-gated ion channels that mediate fast excitatory synaptic transmission in the mammalian CNS (Wisden & Seeburg, 1993; Hollmann & Heinemann, 1994; Dingledine *et*

al., 1999; Erreger *et al.*, 2004; Traynelis *et al.*, 2010). The channel pores are formed from four of one or more subunits (AMPA: GluA1–4; kainate: GluK1–5), each of which has an amino terminal domain, a glutamate-binding domain, a transmembrane domain and an intracellular carboxyl terminus (Gouaux, 2004; Sobolevsky *et al.*, 2009; Traynelis *et al.*, 2010). Homomeric or heteromeric assemblies of pore-forming subunits form functional receptors, but native receptors also have auxiliary subunits that modulate their gating behavior and/or localization (Jackson & Nicoll, 2011; Straub & Tomita, 2012; Yan & Tomita, 2012).

The first such auxiliary subunit identified was TARP γ -2 (stargazin), which is one member of a family of proteins, the transmembrane AMPA receptor regulatory proteins or TARPs (Hashimoto *et al.*, 1999; Chen *et al.*, 2000; Brecht & Nicoll, 2003; Tomita *et al.*, 2003; Kato *et al.*, 2008). Several groups have shown that type 1 TARPs (γ -2, γ -3, γ -4, γ -8) modulate receptor responses to glutamate in an isoform-specific manner (Priel *et al.*, 2005; Tomita *et al.*, 2005; Turetsky *et al.*, 2005; Cho *et al.*, 2007; Korber *et al.*, 2007; Kott *et al.*, 2007; Milstein *et al.*, 2007; Suzuki *et al.*, 2008; Milstein & Nicoll, 2009; Jackson *et al.*, 2011). Kainate receptors also have auxiliary subunits (Copits & Swanson, 2012; Straub & Tomita, 2012; Tomita & Castillo, 2012; Yan & Tomita, 2012). The proteins neuropilin tolloid-like 1 and 2 (Neto1, Neto2) are single transmembrane subunits that modulate the gating kinetics of recombinant and native kainate receptors (Zhang *et al.*, 2009; Straub *et al.*, 2011a,b; Tang *et al.*, 2011; Fisher & Mott, 2012, 2013).

Modal gating, where receptors switch on a slow time-scale between kinetically distinct behaviors, has been characterized for *N*-methyl-D-aspartate (NMDA) receptors (Maki *et al.*, 2012; Murthy *et al.*, 2012; Popescu, 2012; Vance *et al.*, 2013) and shapes population responses to rapid agonist applications (Popescu & Auerbach, 2003; Popescu *et al.*, 2004; Zhang *et al.*, 2008a; Vance *et al.*, 2012). Modal gating has also been reported for AMPA receptors (Poon *et al.*, 2010, 2011; Prieto & Wollmuth, 2010), and single-channel studies have characterized the effects of type I TARPs and Neto2 on unitary currents (Tomita *et al.*, 2005; Zhang *et al.*, 2009; Shelley *et al.*, 2012).

Here we have studied single-channel currents evoked repeatedly by fast glutamate applications to patches containing only a few receptors. The results show that type 1 TARPs and Neto2 promote gating where the open probability (P_{open}) is much higher than for receptors without their auxiliary subunits. Because this behavior occurs infrequently and is long lasting, we refer to it as modal gating. Although switching between modes occurs on a time-scale of seconds, it impacts significantly ensemble currents evoked by brief applications of glutamate.

Materials and methods

tsA201 cells were maintained and transfected as previously described (Robert *et al.*, 2001). All GluA plasmids used were flip splice variants. Individual GluA and TARP clones were co-transfected at ratios of 1 : 3. GluK2 and Neto2 were co-transfected at a ratio of 1 : 10. Chimeras in which the first extracellular domain (from D31 to S105 for γ -2, and D31 to S108 for γ -4) was exchanged between γ -2 and γ -4 were constructed with polymerase chain

reaction, as were tandem constructs in which the C-terminus of GluA1 or GluA4 was directly fused to the N-terminus of γ -2 (Cho *et al.*, 2007; Morimoto-Tomita *et al.*, 2009). The GluA1_ γ -2(γ -4Ex1) tandem was made by replacing γ -2 in the GluA1_ γ -2 tandem construct with a chimeric γ -2 with the first extracellular domain of γ -4 by restriction digestion followed by ligation. Putative chimeric and tandem constructs were selected by diagnostic restriction digests and verified by sequencing.

Recordings from outside-out patches were performed 1–3 days post-transfection at room temperature with an EPC-9 amplifier (HEKA) as described (Robert & Howe, 2003). All recordings were made at a holding potential of -100 mV. The external solution contained (in mM): NaCl, 150; KCl, 3; CaCl₂, 2; MgCl₂, 1; glucose, 5; HEPES, 10 (pH 7.4). Patch pipettes (open tip resistance 4–10 M Ω) were filled with a solution containing (in mM): KF, 120 or CsF, 135; KOH, 33 or CsOH; MgCl₂, 2; CaCl₂, 1; EGTA, 11; HEPES, 10 (pH 7.4).

Glutamate (10 mM) was added to the external solution, and was applied with theta glass pipettes mounted on a piezoelectric bimorph (Robert & Howe, 2003). Voltage pulses were applied to the bimorph with protocols controlled by the PULSE (HEKA) software used to acquire the data. The rate of solution exchange (estimated from open-tip potentials) was 100–200 μ s. The bath was superfused constantly with normal external solution flowing at a rate of 1 mL/min.

Single-channel currents were recorded and analysed as described previously (Zhang *et al.*, 2008b, 2009). Briefly, glutamate-evoked currents were analog low-pass filtered at 10 kHz, sampled at 25–40 kHz, and written directly to the hard drive of two computers. One computer stored the records in PULSE format (the software used to run the EPC 9) and the other stored the data in QuB (<http://www.qub.buffalo.edu>) format. Single-channel data recorded during continuous agonist applications were subjected to digital Gaussian low-pass filtering at 3 or 4 kHz (-3 dB), which resulted in closed-channel r.m.s. noise values of 300–350 fA. The closed-channel current was manually set to zero, and the entire filtered data set from a given patch was idealized with the segmental k-means (SKM) algorithm of QuB to identify single-channel transitions, and estimate conductance levels and event durations. The time resolution was set at two or three sample intervals. Both AMPA and kainate receptors display frequent subconductance states (Swanson *et al.*, 1996; Rosenmund *et al.*, 1998; Smith *et al.*, 1999; Jin *et al.*, 2003). The kinetic model used for the SKM idealization of the results contained two closed states and one open state for each of the four (AMPA receptors) or three (kainate receptors) open levels found in each of the records (Smith & Howe, 2000; Zhang *et al.*, 2008b, 2009). The states were connected so that all types of transitions between the five different conductance levels were allowed. Conductance levels, open and shut times, and burst durations were analysed in QuB.

Rapid applications of 10 mM glutamate (100–600 ms) were repeated 142–640 times, at intervals sufficiently long to allow recovery from desensitization between trials. Only patches that did not exhibit rundown of receptor activity were used for the analysis reported here. The entire sequence of trials was analysed for apparent non-randomness using the Runs test (Horn *et al.*, 1984; Howe & Ritchie, 1992). Individual trials were designated low- P_{open} or high- P_{open} based on the following criteria. For AMPA receptors, trials with bursts >

8–10 ms in duration were designated as high- P_{open} . These values were chosen from inspection of the distribution of all bursts in the records (bursts were defined as a series of open periods, to any open level, that were separated by closings briefer than 2–3 ms on the basis of the distribution of closed times) to distinguish short and long burst components seen with TARPs (Tomita *et al.*, 2005). For GluK2, in addition to doubling the mean duration of bursts, Neto2 co-expression also results in clusters of bursts that persist long into the glutamate application (Zhang *et al.*, 2009). For the GluK2 + Neto2 receptors studied here, trials were designated high- P_{open} if bursts longer than 10 ms persisted or occurred at times > 20 ms after the start of the application. Trials that did not contain openings (blanks) were assigned the same value as trials on either side of them if the latter values were the same. If trials bounding blanks differed in their designation, the blanks were assigned alternate values sequentially. In the records analysed, such blanks were rare and their assignment did not substantially affect the number of runs in the record or conclusions regarding statistical significance.

The sequential list of binomial assignments (low- or high- P_{open}) was then analysed for ‘runs’, where a ‘run’ consists of a series of trials of the same type, i.e. trials that exhibit either low- or high- P_{open} gating. We then calculated the Z statistic, where:

$$Z = - [R - 2n(p)(1-p)] / [2\sqrt{n}(p)(1-p)], \quad (1)$$

n is the number of trials, $\sqrt{n}$ is the square root of this number, R is the number of runs, and P and $(1-P)$ are the probabilities of observing the two types of event. The statistic Z has a standard normal distribution, with a mean of zero and a standard deviation of 1, and can be used to determine P -values. The product, $2n(P)(1-P)$, gives the number of runs expected for any data set, and values of Z substantially larger than zero indicate that there are significantly fewer runs than expected for a binomially distributed variable. For example, Z -values of 1.65 and 1.96 correspond to a P -value of 0.05 for one-tailed and two-tailed confidence intervals, respectively. For the quantitative results reported here, n was 123–641, and the number of runs observed, R , was 34–209. Given that the criteria used to sort low- and high- P_{open} trials were based on exponentially distributed events (burst durations), misclassification of trials was inevitable (although the criteria were selected to limit this confounding factor by minimizing the total number of misclassifications). The impact of this on the results for individual patches is impossible to know; however, the values of Z ranged from 2.96 to 19.8 and, with one exception ($Z = 2.96$, $P = 0.0032$), all the patches analysed gave P -values below 0.000025. It therefore seems very unlikely that misclassification altered the significance of the results. In support of this conclusion, the results of the Runs analysis were relatively insensitive to the exact criteria selected, and independent analysis by two separate authors gave similar results.

For AMPA receptors, the Runs analysis was limited to patches that had three or fewer receptors (most had one or two). The number of receptors active in each patch was determined by examining the amplitude of events evoked at the onset of rapid jumps into saturating glutamate. For AMPA receptors, the open probability at the peak of the ensemble average was close to 0.9 and we had hundreds of jumps for each patch analysed. In these

patches, distinguishing whether there were one, two, three or more receptors active was virtually unequivocal. For patches that contained two or three receptors, trials were designated as high- P_{open} if one or more receptors displayed such behavior, and low- P_{open} if none of the receptors displayed high- P_{open} behavior. For kainate receptors, a substantial proportion of the trials did not contain openings, even when multiple receptors were present in the patch. The number of receptors was therefore indeterminate. Despite this ambiguity, however, all patches contained runs of high- P_{open} trials that appeared to arise from the activity of only one receptor, as demonstrated by the rarity of double openings during this activity. Mean ($\pm$ standard deviation) values are reported in the Results.

Results

In addition to their role as chaperones and adaptor proteins that deliver receptors to the cell surface and anchor them at synapses, TARPs modulate the functional properties of AMPA receptors in several important ways. Although different mechanisms for TARP modulation have been proposed (MacLean & Bowie, 2011; MacLean, 2013), the results of our previous work indicate that all four type 1 TARPs (γ -2, γ -3, γ -4 and γ -8) decrease the activation energy required for channel activation by increasing β , the rate constant for channel opening (Tomita *et al.*, 2005; Zhang *et al.*, 2006; Cho *et al.*, 2007). Because glutamate dissociation, channel opening and receptor desensitization all occur from the same set of brief closed states (Raman & Trussell, 1995; Partin *et al.*, 1996; Cho *et al.*, 2007), TARP-mediated increases in β result in more channel openings before either glutamate dissociates or the receptor desensitizes. Modulation by TARPs therefore slows both deactivation and desensitization; and all type 1 TARPs consistently and substantially enhance a slow component of both deactivation and desensitization (Cho *et al.*, 2007; Milstein *et al.*, 2007). This TARP modulation of activation gating and the effect of TARPs to speed recovery from desensitization (Priel *et al.*, 2005) results in less depression of responses to repeated receptor activation when brief pulses of glutamate are applied at intervals of 20–100 ms (Cho *et al.*, 2007).

Type 1 TARPs promote modal gating of AMPA receptors

Consistent with these effects on population responses, at the level of unitary currents co-expression of γ -2 prolongs the duration of bursts of channel openings and increases the proportion of time that AMPA receptors spend at large conductance levels when they are open (Tomita *et al.*, 2005). When fast, sustained, applications of saturating concentrations of glutamate are repeated many times on outside-out patches containing only a few active receptors, the co-expression of γ -2 results in trials with large-conductance activity (openings to levels of 30–45 pS) throughout the applications (Fig. 1A). As expected from the rapid and nearly complete decay of large ensemble currents through receptors assembled solely from pore-forming GluA subunits, such activity is rarely seen in the absence of TARP co-expression (Tomita *et al.*, 2005; Zhang *et al.*, 2008b).

The high- P_{open} , large-conductance gating behavior we saw previously in γ -2 co-expression studies appeared to cluster in runs of consecutive trials, even though the patches contained 5–10 active receptors. To test further the extent to which this clustering was real, we

repeatedly applied glutamate to patches that contained no more than three active receptors. In each of eight patches co-expressing GluA4 and γ -2, high- P_{open} activity tended to occur in runs of consecutive trials. For example, 247 applications of 10 mM glutamate (200 ms) were made at 2-s intervals on the patch shown in Fig. 1A. Although only 35 of the trials contained long large-conductance bursts, the 10 records in Fig. 1A occurred during consecutive applications. Similar clustering of high- P_{open} trials was seen in each of the seven other patches from cells co-transfected with GluA4 and γ -2, as well as each of two patches expressing GluA1 and γ -2, and two patches expressing GluA1 and γ -4, suggesting this may be a general feature of TARP modulation of AMPA receptor gating.

The statistical significance of such clustering, and whether it deviates from binomial predictions, can be assessed with a Runs test (Horn *et al.*, 1984; Howe & Ritchie, 1992). A 'run' consists of a series of trials of the same type, in this case trials that exhibit either low- P_{open} or high- P_{open} gating. The test allows calculation of a statistic, Z , which has a standard normal distribution and can be used to determine P -values (Materials and methods). Values of Z substantially larger than zero indicate that there are significantly fewer runs than expected for a binomially distributed variable. In each of the 11 patches subjected to Runs analysis, the number of runs was substantially less than expected for a random binomially distributed variable ($Z = 4.21\text{--}7.34$, $P < 0.0001$). Our interpretation of this result is that the inclusion of TARPs in receptor complexes promotes a high- P_{open} gating mode that is characterized by long bursts during which the receptor spends significant time at the largest conductance levels. Individual receptors switch between the distinct low- and high- P_{open} gating modes infrequently, and the mean time they spend in each mode is longer than the 2-s intervals that separate consecutive trials. As a result, the likelihood that individual receptors display high- P_{open} gating during any one of a series of consecutive glutamate applications is influenced by what mode the receptor was in during trials that immediately preceded it.

Modal gating of GluA_TARP tandem receptors

We previously presented evidence that glutamate-induced desensitization caused GluAs and TARPs to uncouple functionally, reducing TARP-mediated enhancement of steady-state currents during sustained applications of glutamate and blunting the slow component of deactivation (Morimoto-Tomita *et al.*, 2009). These conclusions were based in part on comparison of currents in co-expression experiments with those for tandem receptors where the intracellular C-terminus of GluA1 or GluA4 was directly linked to the intracellular N-terminus of γ -2. We hypothesized that similar uncoupling might limit TARP-mediated high- P_{open} gating. To test this hypothesis, we recorded single-channel currents evoked by repeated applications of glutamate in patches that contained GluA4_ γ -2 tandem receptors. The experiments on tandem receptors also avoided potential heterogeneity in receptor stoichiometry (in patches with more than one receptor), as the TARP:GluA subunit ratio was fixed at 1 : 1.

The high- P_{open} behavior seen in the γ -2 co-expression studies was even more evident for receptors assembled from GluA_TARP tandems. Figure 1B shows unitary currents evoked by 10 consecutive concentration jumps in a patch with two active GluA4_ γ -2 tandem receptors during a period where one of the receptors was displaying high- P_{open} gating. The

records contain very long bursts, during which the receptor spends most of its time rapidly oscillating between the two highest open levels (34 and 42 pS, note the increased noise when the channel is open).

Figure 2 shows 30 consecutive trials from a patch containing just one GluA4_γ-2 tandem receptor. This portion of the recording contains nine runs, where each run consists of consecutive trials of either low- or high- P_{open} gating, defined, respectively, by the absence or presence of bursts of openings > 10 ms in duration (designations as low- or high- P_{open} trials are indicated by 0 or 1 to the left of each record). Note that some of the burst activity during high- P_{open} trials persists or occurs after the glutamate application terminates. Although the frequency with which such events occurred varied, they were seen in most tandem-receptor patches and did not reflect imperfect solution exchange (as assessed by tip potential measurements). We identified 64 runs in 229 consecutive trials obtained from this patch, whereas the relative frequencies of low- and high- P_{open} gating (0.712 and 0.288, respectively) predicted 94 runs. The Z value of 4.84 obtained from this analysis corresponds to $P < 0.000001$.

We next asked whether the low- and high- P_{open} modes observed at the level of unitary currents correlate with the fast and slow components in the bi-exponential decay of population currents seen with TARPs. Selected examples (not consecutive) of low- and high- P_{open} trials in another patch containing a single GluA4_γ-2 tandem receptor are shown in Fig. 3A and B. Runs analysis of 300 consecutive trials in this patch identified 77 runs compared with 126 expected ($Z = 6.74$). Figure 3C and D shows ensemble currents from low- and high- P_{open} trials. The top traces in Fig. 3C and D are the sum of the last five records in Fig. 3A and B (note the smaller low-mode peak current), and the bottom traces are the corresponding ensemble averages from all the low- and high- P_{open} records (210 and 90, respectively). The single exponential fits to these average currents gave time constants of 2.54 and 14.7 ms. These and similar data from three other one-channel patches gave mean time constants of 3.91 ± 1.02 and 23.7 ± 8.1 ms.

The time constants obtained for low- and high- P_{open} modes from the one-receptor patches agree well with values for the fast and slow components in the bi-exponential desensitization decays of large population currents in patches with many receptors (Cho *et al.*, 2007; Milstein *et al.*, 2007). The corresponding values for GluA4_γ-2 tandem receptors in patches with peak currents of 255–590 pA were 4.00 ± 0.92 and 13.9 ± 5.0 ms ($n = 8$). The relative amplitude of the slow component plus the steady-state current from the fits to these large GluA4_γ-2 population currents (0.407 ± 0.081 , $n = 8$) is in good agreement with the relative proportion of high- P_{open} trials measured in one- and two-receptor patches for tandem receptors (0.365 ± 0.105 , $n = 7$). In the two-receptor patches, the probability of one receptor being in the low- P_{open} gating mode was calculated as the square root of the observed probability that both were in the low- P_{open} mode. The probabilities so obtained predicted well the observed probabilities that both receptors were in the high- P_{open} mode or that one was in low-mode and the other in high- P_{open} mode, as expected if the two receptors gated independently. It is noteworthy that even for the tandem receptors, on average the receptors gate in the low- P_{open} mode about two-thirds of the time, a mode that appears kinetically

indistinguishable from behavior exhibited by receptors formed solely by pore-forming subunits.

Compared with our previous work on patches from cells co-transfected with GluA4 and γ -2 (Tomita *et al.*, 2005), the GluA4_ γ -2 tandem receptors appear to spend more time at large conductance levels. Figure 3E shows single-channel currents during continuous glutamate in another patch and a histogram of the amplitude of events from bursts of receptor activity. The four conductance levels seen for GluA4_ γ -2 receptors in nine patches: 9.2 ± 1.5 pS, 20.3 ± 1.7 pS, 32 ± 2.2 pS, 42.8 ± 1.3 pS agree very closely with values we reported before for homomeric receptors composed of GluA4 subunits (8.7, 19.5, 31.0, 44.8 pS; Tomita *et al.*, 2005), but for the tandem receptors openings to the two largest levels predominated. As shown in Fig. 3F, during receptor activations, this GluA4_ γ -2 receptor spends most of its time at the largest subconductance level. This differs from single-channel results for GluA4 with and without γ -2 where, although γ -2 promotes large-conductance openings, they represent only about 7% of the events in co-expression experiments (Tomita *et al.*, 2005), and it is in stark contrast to GluA2 receptors formed from only pore-forming subunits, which open almost exclusively to the two smallest conductance levels (Zhang *et al.*, 2008b). Although we have not analysed the results in detail, larger-conductance openings seemed more common when the receptor was in the high- P_{open} mode and the peak amplitude of average ensemble currents through GluA4_ γ -2 receptors was 10–20% higher for high- P_{open} trials than the peak amplitude of corresponding currents for low-mode gating.

The first extracellular loop of type 1 TARPs is primarily responsible for isoform-specific differences in modulation of AMPA receptor gating (Cho *et al.*, 2007; Milstein *et al.*, 2007). To begin to characterize modal gating for additional GluA_TARP tandems, we conducted similar studies on patches expressing tandem receptors made with GluA1 and γ -2 ($n = 3$) or tandem receptors [GluA1_ γ -2(γ -4Ex1)] made with GluA1 and a chimeric TARP where the Ex1 domain of γ -4 was swapped into the γ -2 backbone ($n = 2$). All five of these additional patches contained either one or two active receptors, and each patch showed strong evidence of modal gating.

Examples of results obtained from a patch containing two GluA1_ γ -2(γ -4Ex1) receptors are shown in Fig. 4. This patch was particularly stable, and we were able to analyse 641 consecutive 100-ms jumps into 10 mM glutamate (repeated at 400-ms intervals). Figure 4A and B shows consecutive records designated low- and high- P_{open} . As in other patches that contained more than one receptor, records with multiple openings were designated high- P_{open} provided one or more receptors displayed such behavior. Figure 4C shows the ensemble averages from each group. Note that the peak current is about 50% larger for the high- P_{open} average, which appeared to primarily reflect the greater proportion of large-conductance openings during high- P_{open} gating. Figure 4D shows the ensemble currents normalized to the same peak amplitude. The low- P_{open} average is fitted well by a single exponential with no steady-state component. The high- P_{open} average decays more slowly and has a substantial steady-state component. The lower right corner shows the fit to the ensemble average of all 641 trials. The bi-exponential fit gave time constants and steady-state current similar to values we reported for this TARP chimera in co-expression experiments (Cho *et al.*, 2007). The mean conductance levels were similar for both GluA1

tandems to the values we give for GluA4_ γ -2 above, as well as values for GluA4_ γ -4. For example, the largest open level corresponded to a conductance of 40.1 ± 1.0 pS ($n = 3$) for the GluA1 tandems, and was 40.6 ± 1.0 pS for GluA4_ γ -4 ($n = 9$).

Neto2 promotes high- P_{open} modal gating of GluK2 receptors

We previously reported that Neto2 slowed desensitization and sped recovery from it when co-expressed with the pore-forming kainate receptor subunits GluK1, GluK2 and GluK5 (Zhang *et al.*, 2009; Straub *et al.*, 2011a). In single-channel records obtained with GluK2 and Neto2, there was clear evidence of low- P_{open} and high- P_{open} gating that tended to cluster during consecutive trials in long series of rapid glutamate applications. This behavior was at least qualitatively similar to that described above for TARPs and AMPA receptors. To test the significance of this tendency, we performed Runs analysis on data obtained for nine patches from cells co-transfected with GluK2 and Neto2. The records consisted of series of 123–365 consecutive rapid applications (300–500 ms) of 10 mM glutamate applied at 6-s intervals. Individual trials were designated high- P_{open} if burst activity persisted or occurred more than 20 ms into application. Although we obtained patches that appeared to contain only one or two active receptors, they were not ideal for this analysis because the high- P_{open} mode occurred rarely and there were few runs to analyse. The patches chosen for analysis all showed evidence of three-five receptors being open simultaneously at the beginning of the jump, and the patches may have contained twice that many receptors.

Examples of the results from one of the nine patches are shown in Fig. 5. Sets of 14 consecutive jumps are shown where none (Fig. 5A) or at least one (Fig. 5B) of the receptors displayed high- P_{open} gating. Figure 5C shows portions of records marked with asterisks in Fig. 5A and B on an expanded time-scale to show the subconductance levels we reported before, which are unchanged by Neto 2 (Zhang *et al.*, 2009). Figure 5D shows the respective ensemble averages from 220 low- P_{open} and 72 high- P_{open} trials. As expected from the effect of Neto2 to decrease the rate constant for entry into desensitized states and increase the probability that the receptors open (Zhang *et al.*, 2009), the peak current is larger for the high- P_{open} average. The longer burst durations and the effect of Neto2 to promote recovery from desensitization give rise to a slow component in the decay of the high- P_{open} ensemble current that is similar to the results we reported for patches containing hundreds of receptors.

Analysis of the complete set of 292 consecutive trials obtained from this patch identified 46 runs (107 predicted) and gave a Z-statistic of 9.7 ($P \ll 0.000001$). The results obtained from the other eight patches were similar in all respects to those illustrated in Fig. 5.

Discussion

We show here that association with auxiliary subunits promotes distinct gating behavior of both AMPA and kainate receptors, where the receptors switch between low- P_{open} and high- P_{open} gating on a time-scale of seconds. For AMPA receptors, this behavior is responsible for the slow component of desensitization that becomes evident when pore-forming subunits are co-expressed with type 1 TARPs (Cho *et al.*, 2007; Milstein *et al.*, 2007). The effect of modal gating on AMPA receptor ensemble currents is reminiscent of the similar effects of NMDA receptor modal gating, where it is responsible for the bi-exponential nature of

receptor deactivation (Zhang *et al.*, 2008a). Although we have not studied deactivation here, the effects of type 1 TARPs on deactivation and desensitization are similar (Cho *et al.*, 2007; Milstein *et al.*, 2007), and it seems likely that the slow component of deactivation seen with TARPs, and perhaps the bi-exponential decay of synaptic currents, is the result of modal gating. For kainate receptors, the high- P_{open} mode appears to account for the major effects of Neto1 and Neto2 on receptor desensitization (Zhang *et al.*, 2009; Straub *et al.*, 2011a; Fisher & Mott, 2013).

The modal nature of the gating behavior described here becomes evident when fast applications of glutamate are repeated many times (at intervals just long enough to allow recovery from desensitization between applications) on patches that contain only a few receptors. In such experiments, because individual receptors spend, on average, longer times in each mode than the interval at which the applications are repeated, low- P_{open} and high- P_{open} trials tend to cluster in runs. Although the assignment of any given trial is subject to error (as the distributions underlying the behavior are exponential), the degree of apparent non-randomness we report here is far too statistically significant to arise from poor sorting of the events.

The relationship between the AMPA receptor modal gating we describe and the multiple gating modes detected without TARP co-expression during long cell-attached recordings (Poon *et al.*, 2010, 2011) or in outside-out patches (Prieto & Wollmuth, 2010) is unclear, as the earlier data were recorded in the presence of cyclothiazide, which itself greatly enhances open probability. The way we collected our data does not admit the same type of analysis done in this previous work, but it is noteworthy that both TARP association and membrane depolarization promote gating to large conductance levels (Prieto & Wollmuth, 2010). We have not seen any evidence of the larger conductance levels seen in the presence of TARPs by Shelley and colleagues (Shelley *et al.*, 2012). In our hands, the conductance levels detected with and without TARP co-expression, and for the corresponding tandem receptors, are not significantly different. What does differ is the proportion of time the receptors spend in the larger levels, which is especially enhanced for the GluA_TARP tandems. Even with γ -4, however, the largest openings we see in patches with one-three receptors are to a conductance level not > 45 pS.

The high- P_{open} gating mode appears substantially more prominent with the tandem fusion proteins than it is in the co-expression studies. Even with the tandems, however, many of the applications produce only low- P_{open} gating with short bursts of openings that include small conductance levels and few openings late in the application. The ensemble currents from the low- P_{open} trials show decay kinetics similar to the predominant component seen in many fast application studies without TARPs, and there is little or no steady-state current. The decay kinetics of the ensemble currents from high- P_{open} trials, as well as the size of the steady-state currents, varied for different GluA : TARP combinations, as well as from patch to patch. However, the ensemble currents always decayed mono-exponentially with a time constant in reasonable agreement with the slow component seen in similar co-expression studies for population responses from patches containing hundreds of receptors (Cho *et al.*, 2007; Milstein *et al.*, 2007). The enhanced steady-state current seems to arise both from occasional very long bursts like those illustrated for GluA4_ γ -2 in Figs 1B and 2, as well as

the greater preponderance of bursts late in the application, presumably secondary to TARP-associated speeding of recovery from desensitization (Priel *et al.*, 2005).

It is of course not surprising that the ensemble currents from low- and high- P_{open} trials have different kinetics. Given our sorting criteria, this result was preordained. What was not a foregone conclusion, however, was that each group would show mono-exponential behavior and that the distribution of individual trials would show evidence of switching between the two types of behavior on such a slow time-scale. The clear presence of modal gating for the tandem receptors eliminates the possibility that it arises from receptors with different GluA : TARP stoichiometries. Although the more prominent high- P_{open} mode seen with the tandem receptors (especially the very long bursts) suggests that desensitization-induced GluA-TARP uncoupling may limit the frequency and duration of high- P_{open} gating, in all patches studied here low- P_{open} trials occurred with substantial frequency in the tandem experiments, even when two or three receptors were active in the patch. We believe our single-channel data with the tandem receptors are consistent with the ideas that TARPs primarily increase the rate constants for channel opening and recovery from desensitization, and that during sustained glutamate applications some receptors are functionally coupled with TARPs and others are not (Suppl. Fig. 5; Morimoto-Tomita *et al.*, 2009). However, the results may also be interpreted to indicate that the high- P_{open} mode arises from a set of conformations entirely distinct from those seen in the absence of TARPs, and that TARPs simply promote access to that distinct set of conformations (Landes *et al.*, 2011; MacLean, 2013). As noted, the low- P_{open} gating mode appears largely indistinguishable from behavior seen in the absence of TARPs, and our observation that the receptors switch between distinct gating behaviors on a time-scale of seconds suggests that the free energy barrier governing transitions between modes is large.

For both AMPA and kainate receptors, the high- P_{open} modes seen in co-expression experiments with the respective auxiliary subunits are still relatively rare, and it might be tempting to ignore this behavior in long continuous recordings. It should be noted, however, that the high- P_{open} modes not only influence the decay of ensemble currents but also their peak amplitude. For AMPA receptors, the peak open probability is high even in the absence of TARPs, but the effect of TARPs to enhance large-conductance openings weights the contribution of the slow component. For kainate receptors, the effect of Neto2 to decrease substantially the rate at which the receptors desensitize increases the probability that individual receptors open in response to rapid applications of glutamate, a result we reported before (Zhang *et al.*, 2009), and which we confirm here. The reduced number of ‘failures’, as well as the longer burst durations, seen with Neto2 results in substantial enhancement of the peak amplitude of ensemble currents.

In conclusion, although modal gating occurs on a slow time-scale, it significantly influences the amplitude and kinetics of responses to glutamate on a time-scale that is relevant to fast synaptic transmission, and it may contribute to the bi-exponential decay of AMPA receptor synaptic currents. While the slow switching between modes makes it unlikely that individual receptors will switch during a single synaptic event, the relative proportion of receptors in each mode will vary from excitatory postsynaptic current to EPSC, especially at synapses where receptor number is small. At any given instant, TARP-associated modal gating results

in a ‘functionally’ heterogeneous population of AMPA receptors, and the shape of the population current reflects that heterogeneity.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
EPSC	excitatory postsynaptic current
Neto	neuroligin tolloid-like
NMDA	<i>N</i> -methyl-D-aspartate
SKM	segmental <i>k</i> -means
TARP	transmembrane AMPA receptor regulatory protein

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Fig. 1.

TARPs promote high- P_{open} gating. (A) Currents evoked by rapid 250-ms applications of 10 mM glutamate in an outside-out patch from a cell co-expressing GluA4 and γ -2. The applications (bars) were repeated at 2-s intervals. The patch contained three active receptors, and peak currents at the beginning of the jumps are off-scale. The large-conductance single-channel activity shown occurred in 10 consecutive trials. The bottom row shows the ensemble average from all 247 trials. (B) Ten consecutive high- P_{open} records evoked by rapid applications of 10 mM glutamate (bar, 500 ms) in an outside-out patch from a cell expressing two GluA4_ γ -2 tandem receptors. The records were obtained sequentially at 2-s intervals. Although there were two active receptors in the patch, in any given trial most of the long bursts of openings to large conductance levels appeared to arise from one receptor, as double openings were rare except at the beginning of the jump into glutamate (off-scale). Note that the receptors step down through the different conductance substates at the end of some of the applications as glutamate dissociates from individual subunits sequentially. Data were low-pass filtered at 3 kHz.

Fig. 2.

TARP-associated receptors switch between gating modes on a slow time-scale. Thirty consecutive records (top to bottom, left to right) from a patch containing one active GluA4_γ-2 tandem receptor. A 500-ms application of 10 mM glutamate (bar above each column of records) was made during each trial, and the trials were repeated at 2-s intervals. Each trial was classified as low- or high- P_{open} based on the absence or presence of a burst longer than 10 ms. The low- and high-mode designations are indicated by a 0 or a 1, respectively, to the left of each trace. This portion of the record contained nine runs, where a run was defined as a series of like-designated trials. The results of Runs analysis on the entire data are given in the text.

Fig. 3.

TARP-associated modal gating results in ensemble currents with distinct decay kinetics and a high proportion of large-conductance openings. (A and B) Selected traces (not consecutive) illustrating low- P_{open} (A) and high- P_{open} (B) gating during 500-ms applications (bars) of 10 mM glutamate in a patch containing a single GluA4_γ-2 receptor. No double openings were observed in this patch during 300 jumps repeated at 2-s intervals. Runs analysis identified 77 runs compared with 126 expected ($Z = 6.74$). Data were low-pass filtered at 3 kHz. (C and D) Top, the sum of the bottom five traces in (A and B) on a faster time-scale. Note the larger peak current in (D). Bottom, average currents from the complete set of low- and high-mode traces (210 and 90, respectively). The decay of each ensemble average is adequately fitted by a single exponential component with the indicated time constants. Note the absence of detectable steady-state current in (C), and the clear presence of steady-state current in (D; dotted line indicates the zero current level). (E) Unitary currents for a GluA4_γ-2 receptor in another patch during part (5 s) of a continuous recording in 10 mM glutamate. Bottom trace shows one long burst on a faster time scale. Data were low-pass filtered at 3 kHz. (F) Histogram and Gaussian fits for the amplitude of events obtained from the SKM idealization of data from 5 min of continuous recording. Four open levels were detected with the indicated conductance levels. Note that the majority of openings are to the 45 pS level.

Fig. 4.

Modal gating influences the shape and size of ensemble currents. (A and B) Examples of unitary currents through GluA1_γ-2(γ-4Ex1) tandem receptors evoked by 100-ms applications (bars) of 10 mM glutamate during low- (A) and high- P_{open} (B) gating (3 kHz filtering). There were two receptors in the patch. The lines to the left of the bottom trace in the right column show the four open levels detected (conductance values indicated). The record contained 208 low- P_{open} and 433 high- P_{open} records [probability of low-mode gating for one receptor = $(208/641)^{1/2} = 0.57$]. Runs analysis identified a total of 61 runs (281 predicted) and gave a Z-statistic of 19.8 ($P \ll 0.000001$). The traces in (C) are the ensemble averages from low- and high- P_{open} trials where only one receptor was active. Note that the peak amplitude of the low- P_{open} average is 60% of the corresponding high- P_{open} peak (1.5 pA vs. 2.5 pA), and high- P_{open} gating accounts for virtually all the steady-state current late in the application. (D) Single-exponential fits to decays of the low- and high- P_{open} averages gave time constants of 3.4 and 13.5 ms. The bi-exponential fit to the ensemble average of all the traces gave time constants for the two components of 5.6 and 18.0 ms. The relative peak amplitudes of the fast and slow components and the steady-state current were 0.37, 0.35 and 0.28, respectively. The dashed lines in (C and D) show the zero-current level.

Fig. 5.

Neto2 promotes high- P_{open} modal gating of GluK2 receptors. (A and B) Two series of 14 consecutive (top to bottom, left to right) applications of 10 mM glutamate to an outside-out patch from a cell co-transfected with GluK2 and Neto2. The applications were 300 ms long and were repeated at 6-s intervals. Based on the unitary currents and the peak currents seen at the onset of the application, the patch contained at least five active receptors. The records were obtained during portions of the recording where none (A) or at least one of the active receptors in the patch (B) was displaying high- P_{open} gating (as defined by the presence of large-amplitude bursts of openings that persisted or occurred at times > 20 ms after the start of the application). (C) Records in (A, top) or (B, bottom) that are marked with asterisks on an expanded time-scale. (D) Ensemble averages obtained from low- and high- P_{open} records (220 and 72, respectively). The low- P_{open} ensemble average had a peak amplitude of 3.75 pA and decayed mono-exponentially with a time constant of 2.6 ms. The mean ensemble current from the 72 high- P_{open} records decayed bi-exponentially and showed a prominent slow component ($\tau_2 = 175$ ms). The peak amplitude of the ensemble average obtained from the high- P_{open} records (4.9 pA) was 30% larger than the corresponding value for the ensemble average of the low- P_{open} trials, although in any given trial only one receptor appeared to exhibit high- P_{open} gating.