Ion channel properties of a protein complex with characteristics of a glutamate/*N*-methyl-D-aspartate receptor

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Abstract **The functional reconstitution of glutamate receptor proteins purified from mammalian brain has been difficult to accomplish. However, channels activated by L-glutamate (L-Glu) and *N*-methyl-D-aspartate (NMDA) were detected in planar lipid bilayer membranes (PLMs) following the reconstitution of a complex of proteins with binding sites for NMDA receptor (NMDAR) ligands. The presence of glycine was necessary for optimal activation. A linear current-voltage relationship was observed with the reversal potential being zero. Channels activated by L-Glu had conductances of 23, 47 and 65 pS, and were suppressed partially by competitive and fully by non-competitive inhibitors of NMDARs. Magnesium had little effect on the reconstituted channels.**

Key words: Glutamate receptor; Ion channel reconstitution; Planar bilayer membranes; NMDA activation

1. Introduction

Activation of ion channel-forming L-glutamate (L-Glu) receptors leads to fast excitatory synaptic transmission in the central nervous system. A large amount of knowledge has been obtained about the structure and function of glutamate receptor proteins that form L-Glu-gated ion channels through cDNA cloning and functional expression of the cloned proteins (for reviews see [1–3]). However, the functional reconstitution of L-Glu receptor-ion channels purified from mammalian brains has been reported by only one group [4,5]. The reported reconstitution did not lead to a separation of different forms of the L-Glu receptors such as the kainate, D,L-α-amino-3-hydroxy-methylisoxazole-4-propionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA) receptors, i.e. there was an incomplete purification of receptor subtypes. The difficulty encountered in the purification and reconstitution of L-Glu receptor proteins is due, in part, to the low levels of extraction of the major subunits of these receptors. For example, the NMDA receptor protein NMDAR1 and the AMPA receptor protein GluR1 are particularly difficult to extract from neuronal membranes [6,7]. Despite the difficulties encountered in isolating substantial quantities of functional complexes of some L-Glu receptor proteins, a complex of four proteins that has ligand binding sites characteristic of NMDARs has been purified from brain synaptic membranes in reasonable yield [8].

This complex is highly enriched in an L-Glu-binding, a carboxypiperazinylphosphonate (CPP)-binding, and a Gly/thienylcyclohexylpiperidine (TCP)-binding protein [8,9]. However, it does not contain the NMDAR1 protein, the key subunit of NMDAR that is absolutely necessary for the expression of receptor activity in complexes formed of the NMDAR1 and NMDAR2 subunits [10–12]. The cDNAs for the L-Glu-binding and Gly/TCP-binding proteins have no significant homology to any previously cloned L-Glu receptor, L-Glu transport, or L-Glu-metabolizing enzyme proteins [13,14]. Therefore, even though this complex of proteins may be easily purified, its potential function as an ion channel-forming L-Glu receptor is not certain.

The role of the complex of proteins purified by Kumar et al. [8] in neuronal function is still not completely defined, but at least one of the subunits of the complex, the L-Glu-binding protein, has been shown to be involved in L-Glu and NMDA-induced Ca^{2+} influx into neurons and the neurotoxicity that follows [15,16]. Reconstitution into liposomes of partially purified preparations of the complex of proteins described above was shown previously to lead to the appearance of L-Glu and NMDA-activated cation fluxes [17]. The L-Glu and NMDA-activated fluxes were substantially enriched in the partially purified and reconstituted fractions. Cation fluxes are, however, an indirect measure of ion channel activity. Therefore, in the present study, the possible formation of ion channels by the complex of proteins reconstituted into liposomes was measured using voltage clamp procedures following the fusion of liposomes with PLMs.

2. Materials and methods

2.1. Preparation of the lipid-reconstituted complexes

Rat brain synaptic membranes were isolated as described previously [8]. A partially purified preparation of the complex of proteins was obtained by one of two methods, batch affinity chromatography of detergent solubilized membrane proteins on L-Glu-derivatized glass fibers [17], or chromatography on L-Glu-derivatized ReactiGel matrices [8]. The solubilization of the synaptic membrane proteins for each of these preparations was performed as described in the respective publications. A more highly purified preparation of the complex was obtained by further chromatographic separation on Sephacryl S-400 HR of the proteins isolated through chromatography on the L-Glu-derivatized ReactiGel [8]. A mixture of dog brain phosphatidyl-

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ethanolamine/cholesterol (4:1 w/w) was introduced in the solubilization and elution buffers to a final concentration of 0.5 mg/ml [17]. All fractions used for reconstitution into lipid bilayers were dialyzed for 2–7 days against a 20 mM potassium acetate, 0.1 mM EDTA, 10 mM Tris-H_2SO_4 buffer, pH 7.4. They were subsequently concentrated to a final lipid concentration of approximately 10 mg/ml.

2.2. Formation of PLMs and protein reconstitution

PLMs were formed and utilized essentially as described by Montal [18]. The final composition of the buffer in the two chambers of the cell separated by the bilayer was: 5 mM HEPES, 150 mM NaCl, 0.2 mM $CaCl_2$, 5 μM Gly (unless otherwise indicated in Section 3), pH 7.6. After formation of a stable bilayer, membrane currents under voltage clamp were recorded via Ag/AgCl electrodes connected to a List/Medical EPC-7 integrated patch-clamp amplifier (gain setting of 50–100 mV/pA). The signal was low-pass filtered at 1–2 kHz, and subsequently digitized at 5–20 kHz. For the majority of recordings, the input headstage electrode of the EPC-7 was placed in the *cis* chamber. The *cis* chamber also contained the proteoliposome-reconstituted protein fractions, while ligands used to activate or inhibit L-Glu receptors were added to the medium in the *trans* chamber held at virtual ground. In this configuration, an applied potential polarizes the membrane in the same direction as that of a neuronal membrane, i.e. the *trans* chamber represented the extracellular side of the membrane. All ligands for the L-Glu receptors were obtained from either Tocris Neuramin or Sigma.

3. Results and discussion

3.1. Activation of ion channels in reconstituted preparations

Liposomes lacking the protein complex did not exhibit any responses to L-Glu or NMDA following fusion with the PLMs. In addition, in all studies with liposomes that contained either the partially or more highly purified preparations of the complex, events characteristic of channel openings were seldom, if ever, detected when either L-Glu or NMDA were absent from the *trans* chamber (data not shown). On the other hand, the presence of either L-Glu (300 nM to 500 μM) or NMDA (20–200 μM) in the *trans* chamber produced transient conductance changes across the PLM when the potentials were held at either positive or negative values. These conductances were typical of single channel openings (Fig. 1). All measurements of L-Glu or NMDA activation of reconstituted proteins were made in the presence of Gly (5–10 μM). Activation by NMDA of ion conductances in reconstituted bilayers was not observed in nominally Gly-free buffers. The ion channel activity produced by the introduction of NMDA required the presence of high concentrations of this agonist (50–200 μM) as well as elevated concentrations of Gly (5–10 μM). The activation of ion channel conductances by L-Glu was only very infrequently observed in nominally Gly-free media and such sporadic events probably were a reflection of both the variability in the concentration of contaminating free Gly in the buffers used during purification and reconstitution, as well as the higher sensitivity of the reconstituted proteins to L-Glu as compared with NMDA. The introduction of Gly alone at 5–10 μM did not usually produce any ion channel-like events.

The purity of the preparation of proteins used in the reconstitution studies did not alter the results obtained with either L-Glu or NMDA (Fig. 1a–e). The most highly purified preparation of the complex is that isolated through successive chromatographic separations on L-Glu-derivatized ReactiGel and on Sephacryl S-400 HR [8]. L-Glu and NMDA activated currents in both the partially (Fig. 1a,d) and the more highly purified (Fig. 1b,c,e) preparations of the complex. Currents activated in the different preparations were similar in terms of their conductance characteristics but differed in their kinetic properties. Channel openings tended to occur in bursts separated by long quiescent periods in some of the more highly purified preparations of the complex reconstituted into PLMs (e.g. Fig. 1 b,c). Such channel kinetics for other receptors have been interpreted as being a reflection of channel cycling through closed, open, and desensitized states [19]. It is possible that prolonged handling of the proteins during extensive purification schemes renders the channel-forming proteins more likely to assume a state that leads to a desensitized channel.

All of these preparations were shown by immunoblot analyses to contain the proteins previously identified in this complex [8], but did not contain any detectable amounts of NMDAR1 (data not shown). The preparations of proteins used in these and other studies have been examined by Western blot analysis for the possible presence of NMDAR1 using two antibodies, a monoclonal anti-NMDAR1 antibody that recognizes all isoforms of this protein and a polyclonal antibody to the carboxy terminus of the NMDAR1a protein (see [8]). As demonstrated in detail in earlier studies [8,9], the detergent solubilization procedure used to extract the complex of proteins that contains the L-Glu, CPP, and Gly/TCP-binding proteins does not extract any of the NMDAR1 protein.

No channel activation was apparent when either AMPA or kainate (10–100 μM) were introduced into the *trans* chamber (data not shown). These two agents define a different class of L-Glu receptors, the AMPA/kainate receptors [20]. Both AMPA and kainate were studied in PLMs that had already exhibited ion channel-like responses following the introduction of either L-Glu or NMDA. The L-Glu and NMDA were removed by continuous perfusion of the *trans* chamber prior to the addition of AMPA or kainate. A similar approach of removing and re-introducing either L-Glu or NMDA did not alter the activation of ion channel events by these agonists. The lack of AMPA activation might be explained as being the result of rapid desensitization of AMPA receptors by this agonist [21]. However, L-Glu also causes rapid desensitization of AMPA receptors, yet the response of the reconstituted complexes to L-Glu was not altered either during long periods of recording or following removal and re-introduction of L-Glu. In addition, kainate activation of AMPA receptors is not characterized by rapid desensitization [21,22]. Thus, it is quite likely that the reconstituted preparations examined did not contain AMPA/kainate-sensitive ion channels. The less selective receptor-ion channel activators, ibotenate and quisqualate, when each

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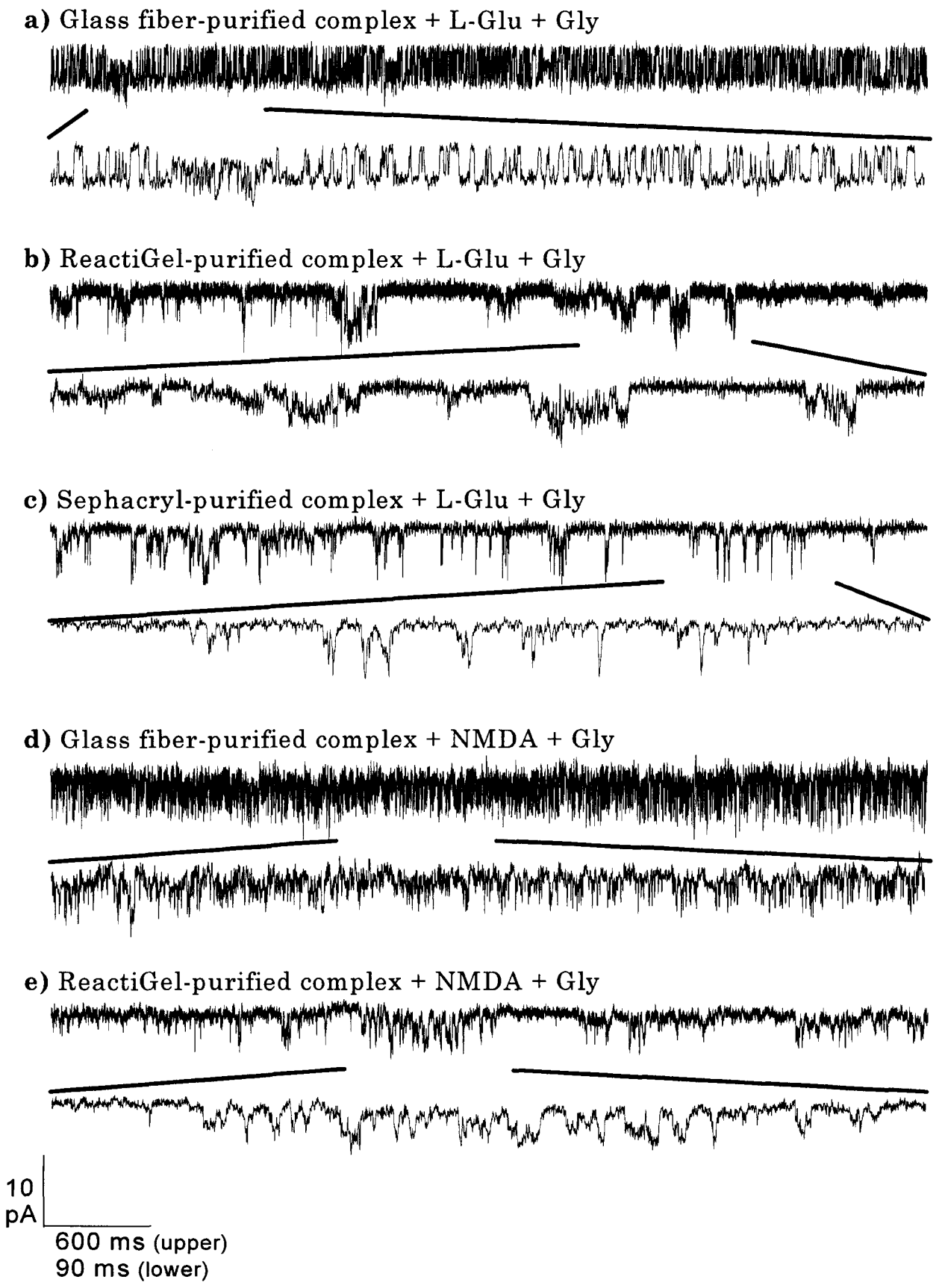
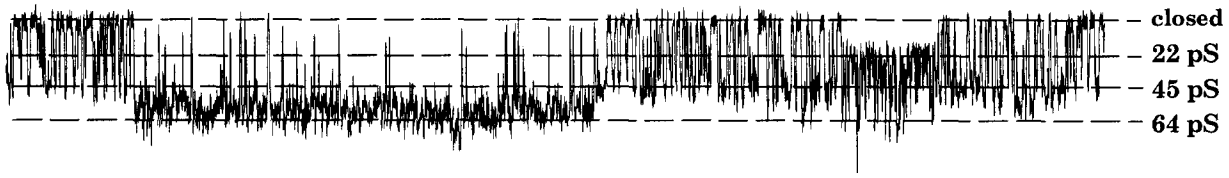
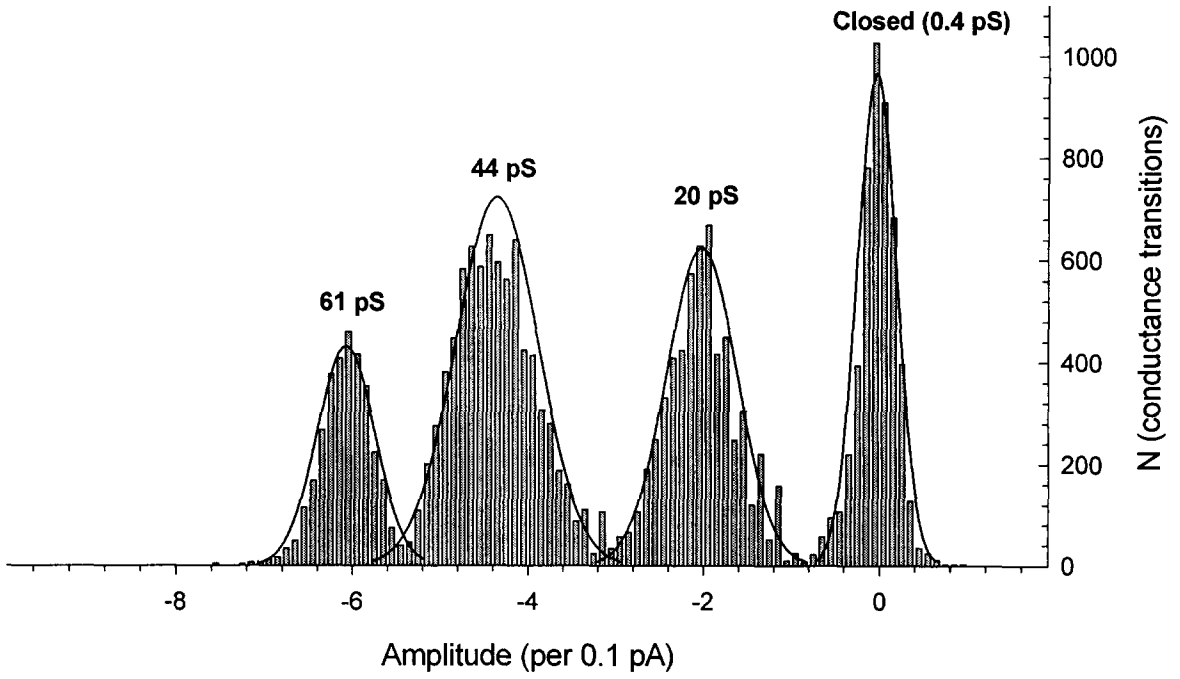
Fig. 1. L-Glu and NMDA activation of ion channel events in PLMs reconstituted with the isolated complex of proteins. Representative current vs. time recordings from PLMs to which reconstituted liposomes were fused and clamped at −100 mV are shown. The type of protein preparation used in the reconstitution experiments and the receptor ligands employed to activate the currents are shown at the top of each tracing. Both time-compressed (5 s, upper tracing) and time-expanded (1 s, lower tracing) records for each condition are shown. Ion channel activity in PLMs reconstituted with different preparations of the isolated complex of proteins was induced either by 10 μM L-Glu plus 5 μM Gly (a,b,c) or by 200 μM NMDA plus 10 μM Gly (d,e).


a) Glass fiber-purified complex + L-Glu + Gly
b) ReactiGel-purified complex + L-Glu + Gly
c) Sephacryl-purified complex + L-Glu + Gly
d) Glass fiber-purified complex + NMDA + Gly
e) ReactiGel-purified complex + NMDA + Gly
10 pA
600 ms (upper)
90 ms (lower)

a) Evident multiple conductance states (10μM L-Glu + 5μM Gly; -100mV)



b) Channel event distribution (10 μM L-Glu + 5μM Gly; -100mV)



c) I-V relationship of complex in PLMs (10μM L-Glu + 5μM Gly)

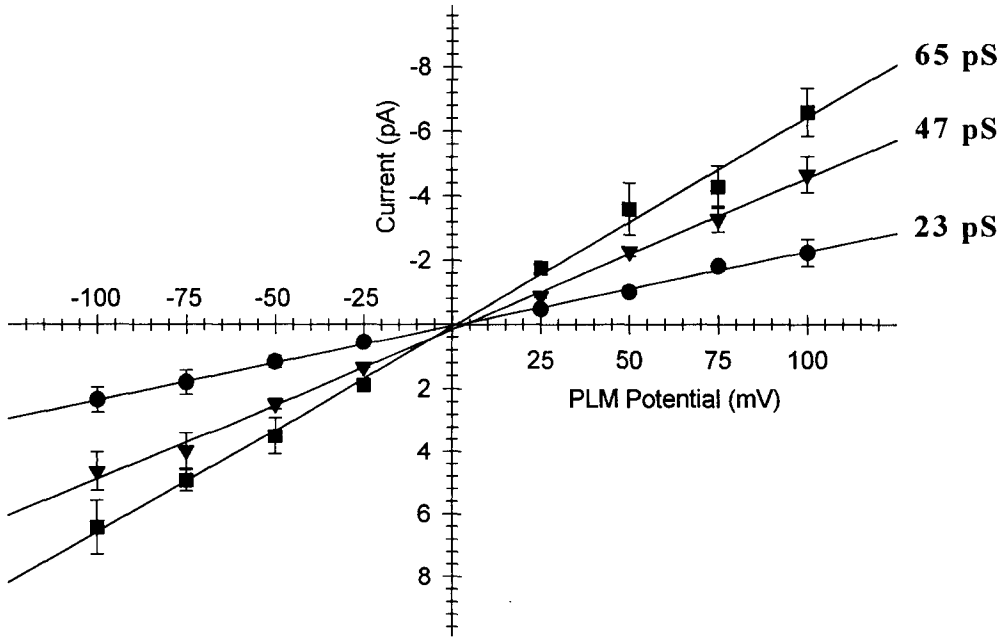


Fig. 2. Conductance properties of the channels activated by L-Glu in PLMs reconstituted with the complex purified through the L-Glu-derivatized glass fiber matrix. The PLM potential was clamped at −100 mV in all experiments shown. a: A representative tracing of channel activity induced by L-Glu and Gly is shown together with identified conductance levels (dotted horizontal lines). The settings for the three conductance levels were compared with those determined by an analysis of the distribution of the 'all-points' amplitude in order to assess their validity. b: Event distribution of channel conductances derived from ~22000 events of transitions of the current amplitude obtained from 4 different activations. The settings for the conductance levels illustrated in a were used for this analysis and the thresholds established for an event were a transition equal to 50% of the amplitude and a duration of 400 μs. The distribution of channel currents was fitted as the sum of four Gaussians with the following means (±S.D.): 0.043±0.3 pA (closed state), 2.0±0.5 pA, 4.4±0.5 pA, 6.1±0.4 pA. c: I-V relationship for the currents activated by L-Glu plus Gly in PLMs reconstituted with the protein complex. The I-V relationships were obtained as described in the text. The slope conductances were obtained from linear regression fitting of the I-V relationships and are shown on the right.
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was present at 10–100 μM concentration, activated channel-like events that were infrequent, of small amplitude, and of very short duration (data not shown). These events were not characterized further.

3.2. Properties of the L-Glu-activated ion channels

Nearly all records obtained following the application of either L-Glu or NMDA to the reconstituted proteins indicated the presence of multiple conductance states (Fig. 1). These events were thought to represent the opening and closing of a single ion channel as transitions from the closed state occurred directly to one of the most frequently observed conductance levels, as did transitions back to the closed state (Fig. 1). However, the possibility that such events were the result of highly cooperative behavior of the reconstituted channel proteins cannot be ruled out. Records or portions of records in which conductance doubling was obvious, i.e. multiple channels were present, were not utilized in the analysis of the characteristics of single channels.

A sample record that illustrates the apparent multiple conductance levels of a single ion channel is shown in Fig. 2a. The cumulative amplitude distribution for single channel events activated by 10 μM L-Glu plus 5 μM Gly is shown in Fig. 2b. The distribution was best fit to a sum of four Gaussians. Recordings from individual experiments indicated that some events with low conductance (< 10 pS) may not have been well resolved due to the background noise. The means of the major conductance levels obtained from the fits to the amplitude distribution of L-Glu-activated channels compiled from different studies performed across different PLM potentials (−100 to +100 mV) were used to construct the current (I) vs. membrane voltage (V) relationship shown in Fig. 2c. The slopes of the lines represent the mean conductance levels for the single channel events. The means (±S.D.) of the conductances for these experiments were 65±6, 47±4, and 23±2 pS. The 47 and 23 pS conductance levels are similar to those for NMDA-activated ion channels in neurons reported by several investigators (e.g. [23,24]). The high conductance state observed (65 pS) may be the result of chelation of free Ca^{2+} and Mg^{2+} by EDTA used in all steps of the isolation and reconstitution of the protein complex. Gibb and Colquhoun [25] have reported the presence of high conductances (> 60 pS) for neuronal NMDARs upon chelation of free extracellular Ca^{2+} and Mg^{2+}. All protein preparations used in the present studies were subjected to extensive dialysis against EDTA. The higher the concentration of EDTA, the

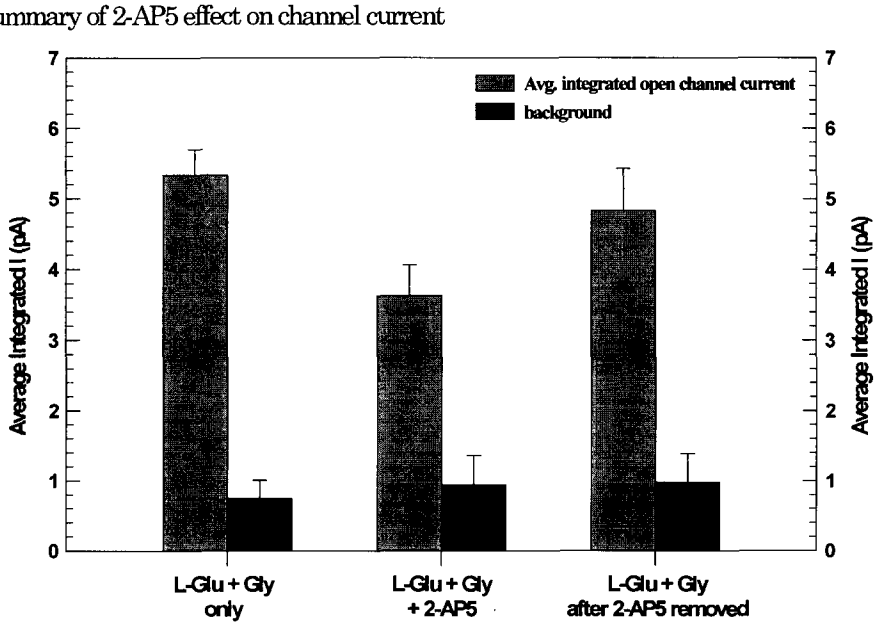

Fig. 3. Partial inhibition by 2-AP5 of the average integrated 'open state' and background ('closed state') current measured in the presence of 10 μM L-Glu and 5 μM Gly in PLMs reconstituted with preparations of the complex. All recordings were obtained at −100 mV applied potential. The integrated current represents the average of 30 s periods of channel activity measured either in the absence or the presence of 200 μM 2-AP5. The effect of 2-AP5 on the channel activity was determined 15 min after the addition of 200 μM 2-AP5. Likewise, the recovery of channel activity was determined 15 min after the removal of 2-AP5.

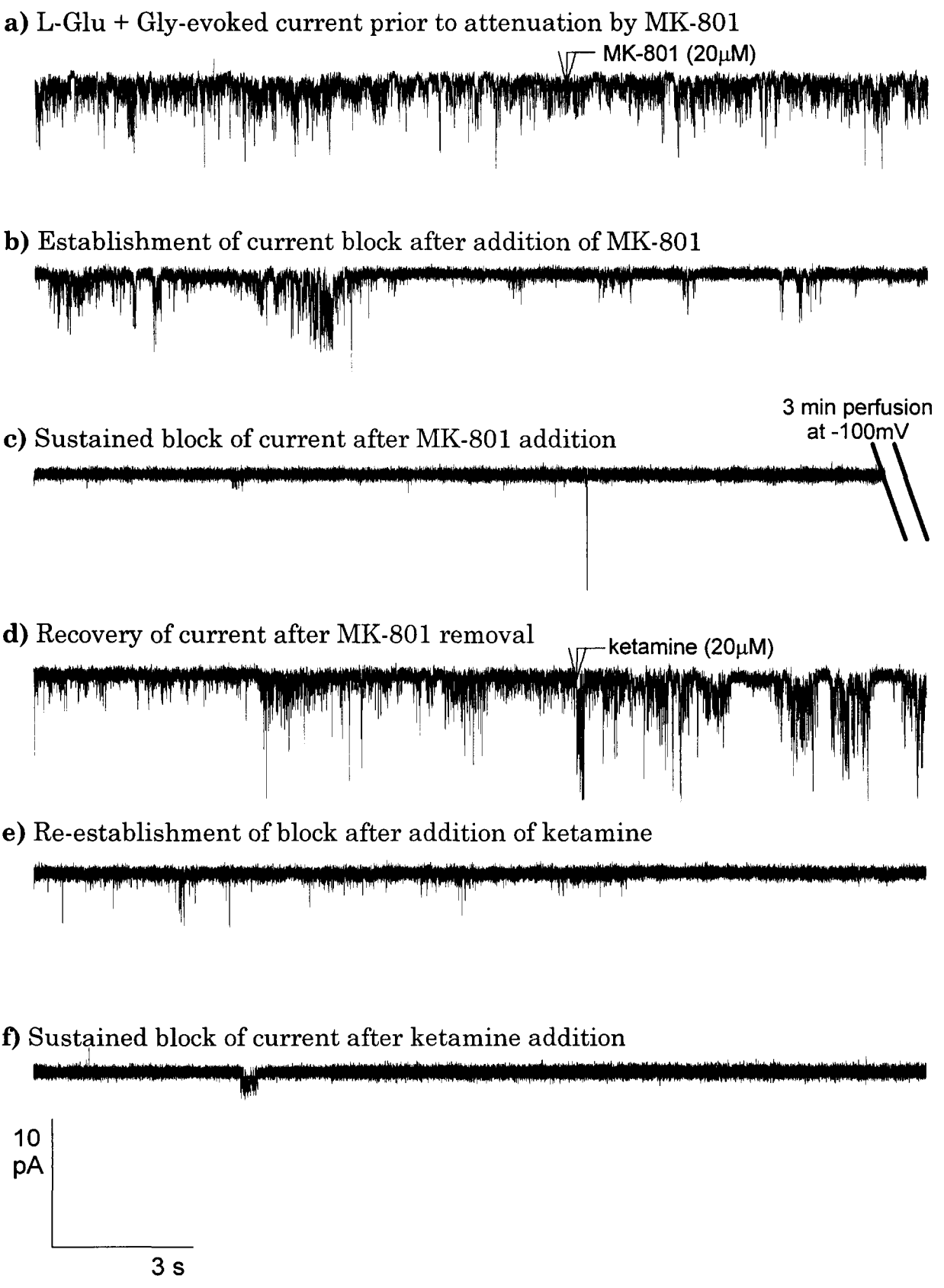
a) L-Glu + Gly-evoked current prior to attenuation by MK-801
MK-801 (20μM)
b) Establishment of current block after addition of MK-801
3 min perfusion
at -100mV
c) Sustained block of current after MK-801 addition
d) Recovery of current after MK-801 removal
ketamine (20μM)
e) Re-establishment of block after addition of ketamine
f) Sustained block of current after ketamine addition
10
pA
3 s

Fig. 4. Inhibition by MK-801 and ketamine of channel activity induced by 100 μM NMDA+10 μM Gly in preparations of the complex reconstituted in PLMs. a,b,c: A continuous record of channel activity, except for the indicated break during the perfusion of the trans chamber, is shown prior to and following the introduction of the channel blocker MK-801. The activity was recorded at an applied potential of −60 mV, but the potential was changed to −100 mV after the perfusion of the trans chamber. d,e,f: Recovery of channel activity induced by NMDA+Gly following the removal of MK-801 and the subsequent inhibition of the channel activity by the introduction of ketamine (20 μM). The applied potential was maintained at −100 mV throughout this period of recording.
←

greater was the observed frequency of channel transitions to the highest conductance states (data not shown).

3.3. Concentration-dependent activation of ion channel events and the effects of receptor inhibitors

Activation of single channel events in reconstituted bilayers was achieved by L-Glu concentrations as low as 300 nM. Analysis of the integrated current of open channel events measured at different L-Glu concentrations at −100 mV and +100 mV potentials were used to construct dose-response curves. The integrated currents reached near maximal values at L-Glu concentrations between 3 and 6 μM; estimates of the K_{act} for L-Glu in different experiments varied between 0.8 and 1.8 μM (data not shown). An estimate for NMDA-induced activation of single channel currents was not obtained.

Since neither AMPA nor kainate induced any channel activation, most of the studies of inhibition of L-Glu or NMDA-activated channels were focused on inhibitors selective for NMDA receptors. The competitive inhibitor 2-AP5 [20] attenuated but did not completely inhibit the channel activity initiated by L-Glu plus Gly (Fig. 3). 2-AP5 decreased primarily the frequency and, to a lesser extent, the duration of channel openings. The integrated current for the 'open state' of the channels was partially reduced, whereas the background current was not (Fig. 3). The 2-AP5-induced inhibition of channel activity was relatively slow, usually requiring 15–20 min for maximal inhibition to occur. Complete inhibition was never achieved, even when concentrations of 2-AP5 as high as 500 μM were introduced into the *trans* chamber. In previous studies [17], ion flux into liposomes reconstituted with the same complex of proteins was strongly inhibited by 2-AP5. In those studies, however, 2-AP5 was added to the proteoliposomes before exposure of the preparation to either L-Glu or NMDA. In the present studies, L-Glu was applied first and was allowed to activate the channel(s) prior to the addition of 2-AP5. It has been reported that once L-Glu is bound to neuronal NMDARs, 2-AP5 is unable to produce complete inhibition of the current activated by L-Glu [26]. It is possible, therefore, that the same slow dissociation of the activator L-Glu took place in the present studies, thus diminishing the activity of 2-AP5 as an inhibitor.

The drugs MK-801 and ketamine are specific inhibitors of the channels associated with neuronal NMDARs [20,27]. Both compounds act at the phencyclidine or TCP binding sites of NMDAR channels. It was previously shown that the interaction of TCP with the isolated complex and with one of its subunits is inhibited by both MK-801 and ketamine [8,9]. In PLMs containing the reconstituted complex, the activation of channels by NMDA plus Gly was suppressed by both MK-801 (Fig. 4a–c) and ketamine (Fig. 4d–f). Removal of MK-801 from the *trans* chamber while continuously applying NMDA plus Gly and maintaining a non-zero holding potential produced a time-dependent recovery of the channel activity (Fig. 4d).

Neuronal NMDARs are also inhibited by Mg^{2+} ions in a voltage dependent manner, i.e. inhibition occurs at potentials more negative than −30 mV and is relieved at potentials more positive than −30 mV [28,29]. The Mg^{2+}-induced block of the NMDAR channels has the characteristics of 'channel flickering' [26]. In the present study, in most reconstituted preparations of the complex it was difficult to demonstrate any consistent effect of Mg^{2+} on the currents activated by L-Glu plus Gly. In a few preparations (approximately 10% of experiments), Mg^{2+} inhibited the channel activity produced by L-Glu, and this effect was observed at both negative and positive membrane potentials. The inhibition produced by Mg^{2+} resulted in nearly complete abolition of current, rather than channel 'flickering,' and it was reversed only at high (>50 mV) positive potentials (data not shown), i.e. the characteristics of Mg^{2+}-induced blockade did not match those of the most frequently studied neuronal NMDARs.

4. Conclusions

The studies described in this paper demonstrate the successful isolation and functional reconstitution of an L-Glu-sensitive receptor-ion channel purified from rat brain synaptic membranes. Based on the results described above, the complex of proteins isolated by Kumar et al. [8] formed L-Glu- and NMDA-sensitive ion channels upon reconstitution into PLMs. The preparations of the complex do not contain immunochemically detectable amounts of the NMDAR1 protein; therefore, it is unlikely that these channels represented the NMDAR channels formed by NMDAR1 and NMDAR2A–D. A more likely possibility is that the complex purified and reconstituted into PLMs is some type of neuronal L-Glu receptor-ion channel that is weakly activated by NMDA and marginally inhibited by Mg^{2+}. It has not yet been demonstrated that the channels described in this paper are present in brain neurons or that they are activated by L-Glu, but this is currently being explored. Highly selective monoclonal antibodies against the L-Glu-binding protein are currently being used to determine the contribution of this protein to both the ion channel activity measured in reconstituted preparations identical to those described above and in intact neurons in primary cultures.

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