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Modulation of an AMPA-like glutamate receptor (SqGluR) gating by L- and D-aspartic acids

E. R. Brown, S. Piscopo, J.-T. Chun, M. Francone, I. Mirabile, and **A. D'Aniello**

Neurobiology Laboratory, Stazione Zoologica 'Anton Dohrn', Naples, Italy

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Summary. L- and D-aspartic acids (L-Asp and D-Asp) are present in the majority of nervous systems. In phylogeny, significant levels have been reported in mollusc brains, particularly cephalopods. To examine the role of L- and D-Asp on a cephalopod receptor, we studied ligand gating of a squid glutamate receptor (SqGluR) expressed in HEK 239 (human embryonic kidney) cells. Under voltage clamp, application of L-glutamate (L-Glu; 1–30 mM), but not D-glutamate (D-Glu), or L- or D-Asp, evoked an inward current of 0.1 nA. L- or D-Asp (200 μM) applied with 20 mM L-Glu, slowed the time course of activation and inactivation of the L-Glu gated current (time constant increased from 1 s (L-Glu alone) to 3 s (D-Asp and L-Glu) and to 19 s (L-Asp and L-Glu)). Our results suggest that in molluscan systems, aspartic acid could act as a neuromodulator during glutamatergic transmission and could significantly alter synaptic integration by slowing glutamate receptor gating.

Keywords: Glutamate receptor – SqGluR – Heterologous expression – L- and D-aspartic acid – Neuromodulation

Introduction

Among the many putative neurotransmitters in animal nervous systems, the amino acid aspartic acid deserves special attention. Unlike 'classic' excitatory amino acids such as L-glutamate (L-Glu), L-aspartic acid (L-Asp) is present in significant concentrations as both D and L isomers. While L-Asp has been demonstrated to represent an agonist at NMDA receptors (Renard and Crepel, 1996), it has been assumed that the D form is likely to be biologically inactive, as it was not considered to be synthesised or metabolised by classic pathways. However a body of work, starting in the 1970s, has shown that L- and D-aspartic acids (L- and D-Asp) are present in significant concentrations in all nervous systems so far examined, and are particularly concentrated in certain marine invertebrates such as cephalopods (where they were first detected, D'Aniello and Giuditta, 1978; D'Aniello et al., 1995). Along with this growing body of evidence, biochemical studies have demonstrated that pathways for synthesis and breakdown are also present (Wolosker et al., 2000). Lastly, the biochemical fractionation of neural tissue into synaptic sub-components shows that D-Asp is present and concentrated alongside the L-form in synaptic vesicles (D'Aniello et al., 1995; Spinelli et al., 2006). At present however there is no direct evidence that D-forms are implicated in synaptic transmission although it has been suggested on the basis of the evidence above that a role in signalling is likely (Spinelli et al., 2006).

To examine the potential role of D-Asp in synaptic signalling, we chose to study the squid nervous system, and in particular the squid giant synapse, as its size and transparency present it as an extremely convenient model to study pre- and post-synaptic interactions. Recently an AMPA – like glutamate receptor (SqGluR) was cloned from this synapse (Battaglia et al., 1993) and we have used it to study the action of glutamate and related amino acids. We have chosen this strategy as a simplifying first step in order to establish the action of D-Asp in the absence of the complicating presence of other receptor subunits, presynaptic effects and access problems at the native synapse. The present results show that neither D- nor L-Asp are capable of gating this channel, however when applied alongside glutamate, both isomers slow significantly glutamate gating of the current.

Materials and methods

Cell culture

HEK 293 (human embryonic kidney) cells were maintained in DMEM (Dulbecco Modified Eagle's Medium, Sigma, Italy) enriched with 10%

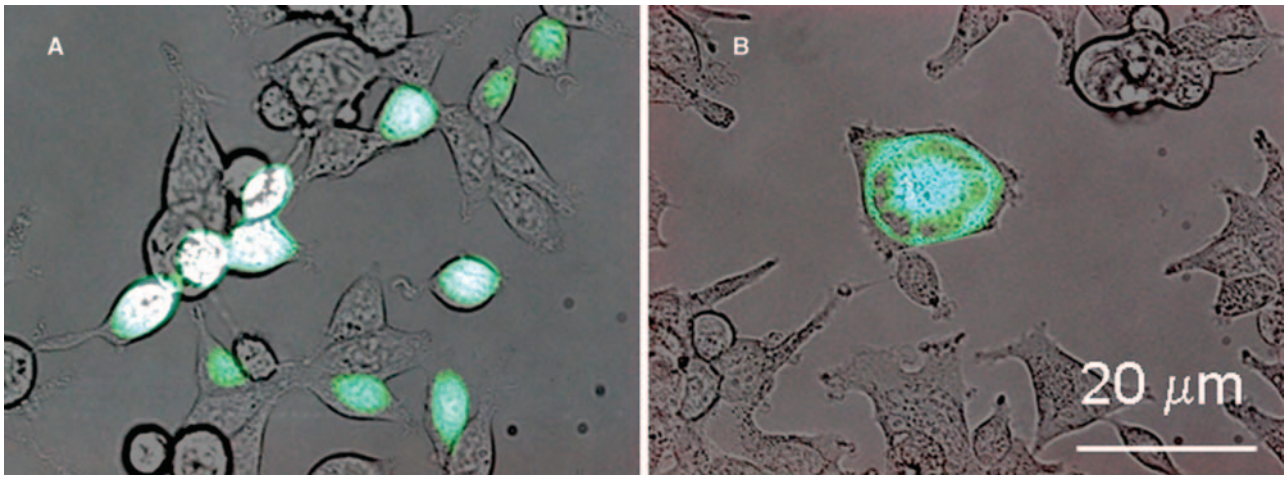
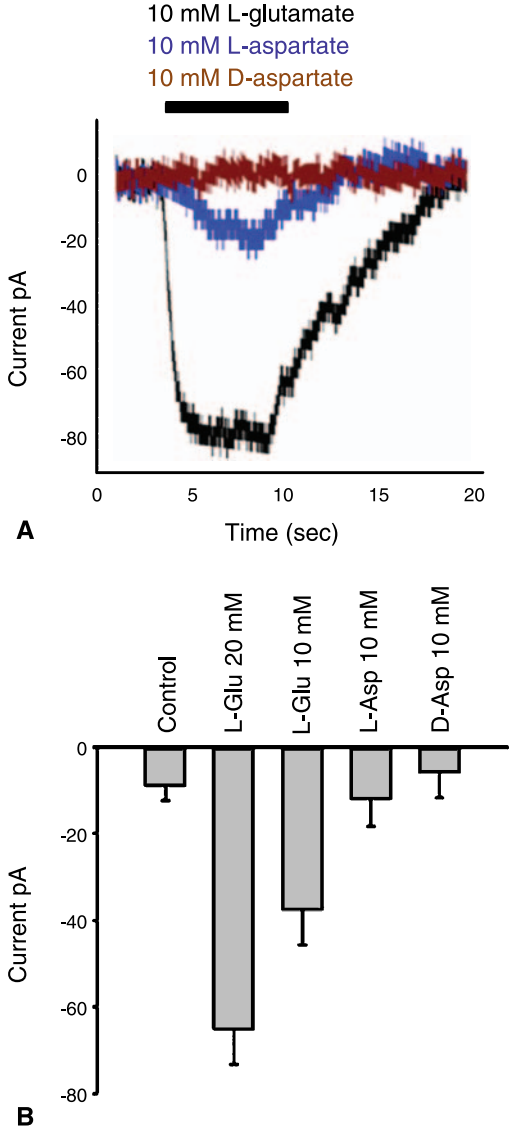


Fig. 1. Expression patterns of GFP and GFP::SqGluR expression in HEK cells illustrated in combined bright-field/fluorescent images. **A** Expression pattern of GFP (control) in HEK cells. **B** Expression pattern of GFP::SqGluR in HEK cells



FBS (fetal bovine serum). Cells were grown until 70–80% of confluence and 24 h before transfection they were treated with Tripsin-EDTA to split them onto cover-slips (pre-treated with poly-L-lysine) inside 60 mm cell culture dishes containing fresh medium. Transfection was carried out with FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) Cells were transfected with a construct containing either a) GFP cDNA (green fluorescent protein) or b) the full length SqGluR clone with GFP cDNA (see below). Around 30% HEK cells expressed GFP ubiquitously and SqGluR::GFP was expressed in a proportion (20%) of the cells and was apparently concentrated in the membranes (Fig. 1).

Molecular biology

The SqGluR clone (short form) was supplied in pox vector courtesy of Dr. W. F. Gilly (Stanford University, USA). To make a plasmid for the over-expression of GluR::GFP fusion protein, the entire coding sequence of the SqGluR cDNA was amplified by PCR. PCR amplification was performed using Hi-Fi Supermix (Invitrogen super mix high fidelity) in 28 thermal cycles with an annealing temperature of 58 °C. The forward (5′-GCTAGC ATGGCTCCAGCCATCGGA CTACCACC-3′) and reverse (5′-GCTAGC CACTTGAGTGT GAGTGTTACCTTC-3′) primers were attached to the *Nhe*I recognition site sequences (underlined) to facilitate the subsequent ligation step. The PCR amplicons were tentatively cloned in pCRII-TOPO vector (Invitrogen), cleaved with *Nhe*I, and eventually ligated to *Nhe*I site of the pQBI-25 vector (Q-Biogene). The sequence of the final plasmid was verified by DNA sequence analysis using the DNA analyser 3730 (Applied Biosystems, USA). The sequences were then compared to the sequence of SqGluR present in Gen-bank EMBL (accession number AJ53463), and errors were corrected with "Quick change site-directed mutagenesis" kit (Stratagene).

Electrophysiology

Glass cover-slips with adherent cells were removed from culture dishes and placed directly in a chamber on an inverted microscope. The chamber was continuously perfused at 2 ml per minute with a standard ringer

Fig. 2. Whole cell voltage clamp recordings showing currents evoked in HEK cells. **A** Currents on application of 10 mM L-Glu, L-Asp, and D-Asp. **B** Summary of results of application of L-Glu and D- and L-Asp. Only application of L-Glu to cells expressing GFP::SqGluR produced significant inward currents

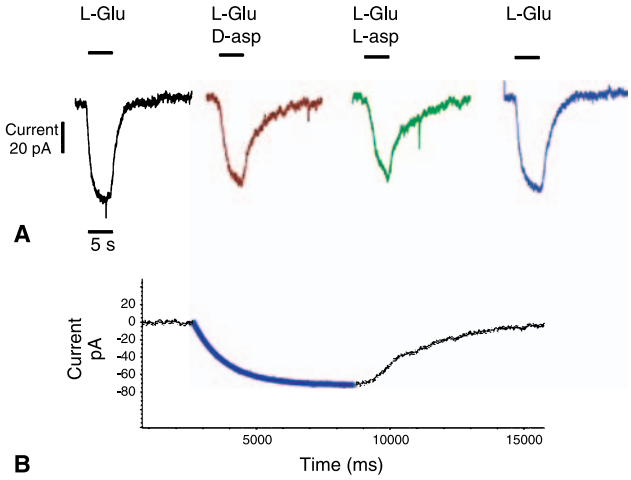


Fig. 3. Effect of 200 µM D- and L-Asp on currents evoked by the addition of 20 mM L-Glu in GFP::SqGluR transfected HEK cells. **A** Examples of currents evoked by L-Glu in the presence of 200 µM D- and L-Asp. **B** Example showing a L-Glu evoked current at a higher time resolution showing the fitting of a single exponential curve to determine the activation time constant

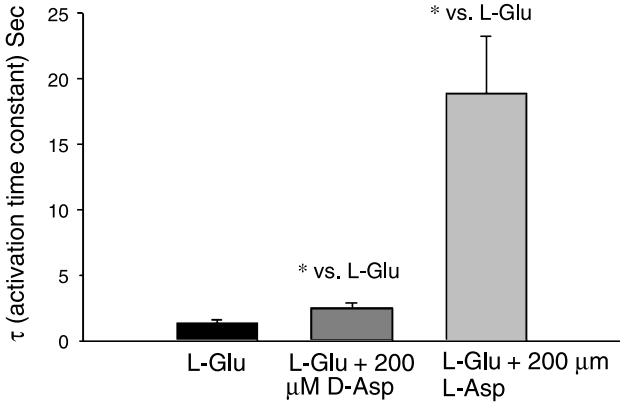


Fig. 4. Comparison of activation time constants of L-Glu gated currents from 6 experiments showing the effect of D- and L-Asp on the time constant of activation of the current in GFP::SqGluR transfected HEK cells. *Significantly different from control $p < 0.05$ (Students paired *t*-test)

solution (5 mM KCl, 135 mM NaCl, 10 mM Hepes-Na, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 25 mM sucrose, in bidistilled water, pH 7.4). Cells were subjected to whole cell voltage clamp. Briefly, 3–5 MΩ pipettes were brought into contact with the cell surface by micromanipulation. GΩ cell attached seals were obtained by gentle suction (1–10 cm H_2O) and whole-cell access obtained by rupturing the GΩ seal patch with direct suction. The internal solution was 120 mM KCl, 20 mM NaCl, 10 mM Hepes-Na, 1 mM $MgCl_2$, and 0.5 mM EGTA, in bidistilled water at pH 7.2. Using an Axoclamp 2B amplifier and a 0.1 Ul headstage (Axon Instruments Inc, USA), capacity transients and feedback resistance were adjusted optimally and the cell clamped to −60 mV. Current and voltage outputs from the amplifier were digitized by a Digidata 1200 digital acquisition system (Axon Instruments Inc, USA) and stored on an IBM PC.

Drug addition

Multiple solution applications were required to compare the effects of D- and L-amino acids along with classical ligands. A 'Valvelink 8' valve perfusion system (Automate Scientific Inc, San Francisco, USA), with 8 solenoid valves which were remotely switched using TTL (Transistor-Transister-Logic) control pulses was used to perfuse cells under voltage clamp. Control pulses were generated using Clampex software (Axon Instruments Inc, USA) through the digital outputs of the Digidata Acquisition system, were amplified to 12 V and delivered to the Valvelink control panel. 10 PSI pressure was applied to the solution reservoirs. The multiple output tubes from the valves led to a 'perfusion pencil' (Automate Scientific Inc, San Francisco, USA), which was an 8 way manifold with a dead-space of around 10 µl. Channel one of the Valvelink system perfused voltage clamped cells continuously with bath solution. Cells were located under ×40 objective and a 100 µM diameter perfusion pipette fitted to the perfusion manifold was micro manipulated to within 300 µm of the cell under voltage-clamp in such a way that the flow from the pipette was against the prevailing current generated by the bath perfusion. It was found necessary to supply a continuous flow of control solution over the cells from the perfusion pipette under resting conditions as sudden changes in perfusion flow (ie. Zero to 10 PSI) caused a pressure artifact on the surface of the cells. Switching times were estimated to be around <100 ms.

Data analysis

Membrane currents evoked by glutamate were analyzed for amplitude and activation kinetics with Clampfit software (Axon Instruments Inc, USA). Current activation rate was calculated by fitting the data with a single exponential function using the formula:

$$F(t) = \sum_{i=1}^{n} A_i^n e^{-t/\tau_i} + C$$

where τ is the activation time constant and C the current as t tends towards ∞. See the curve fit example in Fig. 3B.

Results

Transfection efficiency

Transfection was revealed as a strong GFP fluorescence in a proportion of cultured cells examined. Almost 30% of cells exposed to the GFP transcript were positively labelled after 48 h of incubation. Labelling was apparently evenly spread throughout the cytoplasm (Fig. 1A). In cultures exposed to GFP::SqGluR, the transfection rates of cells were lower than with GFP alone with a rate of around 20% (Fig. 1B). Cells expressing GFP::SqGluR showed a pattern of expression at the cellular level that was different from that of the GFP controls (Fig. 1B). In these cells, the GFP signal was confined to parts of the cytoplasm and was apparently concentrated in a zone near to or in the plasma membrane (Fig. 1B).

Glutamate evoked currents

At a holding potential of −60 mV, L-glutamate evoked an inward current in GFP::SqGluR transfected cells of around 0.1 nA (Fig. 2). No currents were evoked in control cells (either in un-transfected cells or those expressing GFP alone). Reversal potentials were around zero or positive, suggesting that the channel was permeant to Na^+ and

K^+ while channel gating was blocked by 10 μM CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione, data not shown). The effective concentration range of L-Glu was between 10–20 mM (Fig. 2B). Data on the full characterization of this ligand gated ion channel will be presented elsewhere (JT Chun, in preparation).

Aspartate

Application of D- or L-Asp at −60 mV (and at other holding potentials within the range −70 to 60 mV), failed to evoke currents (Fig. 2A, B). Other amino acids or their metabolites were tested (NMDA and D-serine) without evoking currents (data not shown). No current was seen in controls, i.e. in un-transfected cells or in cells expressing GFP alone (Fig. 2B).

When 20 mM L-Glu was applied in the presence of 200 μM of D- or L-Asp, the same peak current was attained as that measured with glutamate alone. However the time taken to achieve these peak values was significantly longer (Fig. 3A). A detailed analysis of the time course of activation of the current was carried out. As described in methods, the activation time constant of the glutamate evoked current was calculated by curve fitting (Fig. 3B). The results of the curve fitting are shown in summary in Fig. 4. It is clear from the data shown in Figs. 3 and 4, that glutamate evoked current activation is significantly slowed in the presence of D- or L-Asp. D-Asp slowed activation by L-Glu threefold while L-Asp slowed activation nineteenfold. Thus L-Asp acid was more potent at slowing receptor activation than D-Asp (Fig. 4). Notably, while the time constant of activation was significantly altered, the peak values remained the same. Quantitatively, this means that gating properties were altered but the total number of channels activated by L-glutamate remained the same. This result and the control experiments rule out the possibility that the slowing effect was due to parallel activation of another current with an opposite current polarity.

Discussion

These results are the first indication that D- and L-aspartic acids are able to directly modulate AMPA-like receptors that are sensitive to L-glutamate. At the same time D- or L-Asp do not gate a current when applied alone, suggesting that if these amino acids are released alongside glutamate during synaptic transmission, postsynaptic potentials could be significantly reduced or delayed. The slowing effect on glutamate current gating could result in either transmission failure or alterations in the capacity for synaptic integration. Since slowing channel gating will produce longer though smaller amplitude synaptic potentials, neurones receiving multiple sources of synaptic information may fire less frequently.

The giant synapse has been studied in detail from the point of view of selectivity of transmission to L-Glu and related substances. Synaptic transmission shows a high selectivity for L-Glu over other amino acids (De Santis and Messenger, 1989). Un-cageing L-Glu evokes postsynaptic potentials similar to these seen in situ (Corrie et al., 1995), and immunological evidence also points towards L-Glu as the putative neurotransmitter at this synapse (Di Cosmo et al., 1999). The receptor subunit we studied is SqGluR, a recently cloned AMPA-like squid glutamate receptor present in the postsynaptic cells of the squid giant synapse, and throughout the cephalopod nervous system (Battaglia et al., 2003). The present study, on the gating of this receptor cloned from the giant axon, further supports the idea that L-glutamate is the excitatory transmitter at this synapse as only L-glutamate gates this channel.

Although the giant synapse of the squid represents a classic ‘escape’ synapse with a high safety factor for synaptic transmission driven by glutamatergic neurotransmission, the action of other potential neurotransmitters or neuromodulators in addition to L-Glu have been reported. Potentially inhibitory ACh (Stanley, 1984) and glycinergic (Vinogradova et al., 2002) responses have been noted, as has potentiation of synaptic transmission by FMRFamide-related peptides (Cottrell et al., 1992). Inhibitory synaptic potentials have also been reported from cells in the stellate ganglion (Miledi, 1972). Could D- or L-Asp act as a neuromodulator at this synapse? It has been reported that no effect on trans-synaptic transmission was seen when D- and L-Asp were applied directly to giant synapses (De Santis and Messenger, 1989). However in this earlier study, drug applications were tested for effectiveness on single trans-synaptic stimuli. Modulatory effects were only seen in other studies during trains of tran-synaptic stimuli (Vinogradova et al., 2002). This means that if there is an action of D- and L-Asp on synaptic modulation, it would be unlikely to be seen during single stimuli. This idea should now be tested in in vitro experiments.

Of course, the discrepancies between this and the earlier studies on the native synapse could also be due to the fact that as in vertebrates, it seems likely that more than one different type of glutamate receptor subunit is assembled heterogeneously to form the native channel complex and that these complexes may behave differently to channel

complexes composed of homomeric subunits (which must be the case in the experiments reported here). In other molluscs such as Aplysia at least 4 putative glutamate (AMPA-like) receptor subunits have been identified (see NCBI accession numbers AAP41203 through to AAP41209). Thus although it has yet to be demonstrated that more than one subunit exists in cephalopods, it seems likely when considering the complexity of these animals, that equal if not greater numbers of subunits will be detected. Thus some caution then is necessary when extrapolating from the behaviour of single subunits to whole synapse behaviour.

The magnitude and time course of the effects of L-Glu and D- and L-Asp are also notable. The maximum current amplitude obtained from the HEK cells on application of glutamate was around 0.1 nA, assuming a similar single channel current as that obtained for mice AMPA glutamate receptors of ∼4 pA (Smith et al., 2000), this indicates that as few as ∼25 active channel complexes are present in the HEK cell membrane. This seems rather a small number of channels and indicates that expression levels were low. Activation and inactivation of the L-Glu evoked current was also much slower than expected. In the giant synapse the postsynaptic potential peaks fully within 2 ms, which is faster than the activation time constant of glutamate receptors at central synapses (3.5 ms; Forsythe and Westbrook, 1988). Activation of cephalopod glutamate receptors by iontophoresis of glutamate appears to be much slower with time constants around 50–60 ms (Lima et al., 2003). In the present case, with valve based perfusion, the activation time constant is apparently an order of magnitude slower again (1s), even if the switching time of perfusion channels was estimated to be around 100 ms (Brown and Piscopo, 2006). Thus it seems likely that this SqGluR subunit has a slow activation time constant and the effect of D- and L-Asp should be tested now on the gating of these receptors at native synapses.

In summary we provide some evidence that D- and L-Asp significantly slow gating of a molluscan glutamate receptor opening the possibility that these substances (at least in molluscs) could act as neuromodulators. These ideas now need to be tested with experiments on native synapses.

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Authors' address: Dr. E. R. Brown, Neurobiology Laboratory, Stazione Zoologica 'Anton Dohrn', Villa Comunale, I-80121 Naples, Italy, Fax: +39 81 764 1355, E-mail: brown@szn.it