Physiological evidence that D-aspartate activates a current distinct from ionotropic glutamate receptor currents in Aplysia californica neurons

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Carlson SL, Fieber LA. Physiological evidence that D-aspartate activates a current distinct from ionotropic glutamate receptor currents in Aplysia californica neurons. J Neurophysiol 106: 1629–1636, 2011. First published July 13, 2011; doi:10.1152/jn.00403.2011.—D-Aspartate (D-Asp) activates an excitatory current in neurons of Aplysia californica. Although D-Asp is presumed to activate a subset of L-glutamate (L-Glu) channels, the identities of putative D-Asp receptors and channels are unclear. Whole cell voltage- and current-clamp studies using primary cultures of Aplysia buccal S cluster (BSC) neurons were executed to characterize D-Asp-activated ion channels. Both D-Asp and L-Glu evoked currents with similar current-voltage relationships, amplitudes, and relatively slow time courses of activation and inactivation when agonists were pressure applied. D-Asp-induced currents, however, were faster and desensitized longer, requiring 40 s to return to full amplitude. Of cells exposed to both agonists, 25% had D-Asp- but not L-Glu-induced currents, suggesting a receptor for D-Asp that was independent of L-Glu receptors. D-Asp channels were permeable to Na⁺ and K⁺, but not Ca²⁺, and were vulnerable to voltage-dependent Mg²⁺ block similarly to vertebrate NMDA receptor (NMDAR) channels. D-Asp may activate both NMDARs and non-L-Glu receptors in the nervous system of Aplysia.

invertebrate; cation channel; reversal potential; buccal ganglion; N-methyl-D-aspartate

IN THE 1960s, D-aspartate (D-Asp) was discovered to have physiological actions in neurons (Curtis and Watkins 1963; Davies and Johnston 1976), but it was not until decades later with the discovery of free D-amino acids in the tissues of several organisms that the relevance of these compounds was understood (reviewed in D’Aniello 2007; Fuchs et al. 2005). Whereas D-serine (D-Ser) has been the subject of much research regarding its role in the nervous system, acting as an agonist at NMDA receptor (NMDAR) channels. D-Asp may activate both NMDARs and non-L-Glu receptors in the nervous system of Aplysia.
contain D-Asp due to high D-Asp racemase immunoreactivity (Scanlan et al. 2010; Wang et al. 2011).

MATERIALS AND METHODS

Cell culture. California sea hares, A. californica (~300–500 g; ~6–8 mo of age and sexually immature with no evidence of mating and no egg masses in the cages), were obtained from the University of Miami National Institutes of Health National Resource for Aplysia (Miami, FL). Primary cultures of BSC cells were prepared according to the methods described by Fieber (2000). Animals were anesthetized for 1 h in a 1:1 mixture of seawater from the culture facility and 0.366 M MgCl₂. Ganglia were then dissected out, and each was placed in a 5-mL solution containing 18.75 mg of dispase (Boehringer Mannheim 1016589001), 5 mg of hyaluronidase (Sigma H4272), and 1.5 mg of collagenase type XI (Sigma C9407) in artificial seawater (ASW) and placed on a shaker set to low speed for ~24 h at room temperature (~22°C). Cells from specific ganglia or areas of ganglia were then dissociated onto 35-mm diameter polystyrene culture plates (Becton Dickinson, Falcon Lakes, NJ) that were coated with poly-d-lysine (MP Biomedicals IC15017525) at 0.2 mg/ml sterile water for 25 min and then rinsed twice in sterile water, dried, and then UV-sterilized before use. Cells were stored at 17°C until used in experiments 24 h later.

Electrophysiology. Whole cell voltage-clamp and current-clamp measurements were made with glass patch electrodes pulled from thick-walled 1.5-mm diameter borosilicate glass capillaries using a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). Voltage and current data were collected, and whole cell capacitance and series resistance compensations were made using an Axopatch 200B clamp amplifier with a capacitance compensation range of 1–1000 pF, connected to a personal computer and Digidata 1200 analog-to-digital converter using pClamp software to record data and issue voltage and current commands (Molecular Devices, Sunnyvale, CA). Bath solutions were flowed onto cells during recording via a 6-bore gravity-fed perfusion system that dispensed solutions from 1-μL micropipettes ~200 μm away from the cell. The environment around the cell was adjusted to a new solution within 500 ms of switching on the flow of the relevant gravity pipette. Solutions containing agonist were briefly applied to the cell via a micropipette tip that was aimed at the cell and positioned at an angle of ~45° from the perfusion flow but closer to the cell, ~30 μm from the cell body. Unless otherwise noted, in all experiments D-Asp was applied via the picrosyringe for 100 ms at a concentration of 1 mM. The effective concentration of agonist was estimated to reach the cell surface ~25 ms after the start of the 100-ms pulse of agonist. Dye-loading experiments indicated that mixing of agonist with the gravity-fed bath solution was minimal for the duration of the picrosyringe pulse due to the proximity of the picrosyringe pipette to the cell and the relatively high force of the picrosyringe pulse compared with the bath solution. To avoid desensitization, ~1–2 min were allowed between applications of D-Asp to individual cells.

To determine the time constants of activation and inactivation, whole cell currents from five cells with comparable current amplitudes were fit using a double-exponential Simplex/SSE algorithm (Axograph). Time constants for these fits were averaged (±SD) across cells.

Desensitization experiments were performed using a two-pulse protocol with the interpulse interval varied from 2 to 44 s. Current amplitude of the second pulse was expressed as a fraction of the initial current. The plot of the normalized average current amplitude as a function of the interpulse duration was fitted using a single-exponential equation: 

\[ I(t) = I_{\text{max}}[1 + (EC_{50}/[\text{agonist}])^{n}] \]

where \( n_{p} \) is the Hill slope coefficient (Verdoorn and Dingleline 1988). Curves and graphs for desensitization and dose-response data were plotted using GraphPad Prism (version 5.03; GraphPad Software, San Diego, CA).

Dose-response data were expressed as a fraction of the current amplitude in response to 10 mM D-Asp. Data were fit using the equation

\[ I(t) = I_{\text{max}}[1 + (EC_{50}/[\text{agonist}])^{n}] \]

where \( n_{p} \) is the Hill slope coefficient (Verdoorn and Dingleline 1988). Curves and graphs for desensitization and dose-response data were plotted using GraphPad Prism (version 5.03; GraphPad Software, San Diego, CA).

Electrophysiological responses of Aplysia neurons to D-Asp. D-Asp-induced currents were present in 54.1% of cultured BSC neurons (n = 37), 28.6% of non-BSC buccal neurons (n = 7), 18.8% of abdominal ganglion bag cell neurons (n = 16), 40.0% of unidentified cerebral ganglion neurons (n = 25), 45.8% of pleural ventral caudal neurons (n = 59), and 31.3% of unidentified neurons of the pleural ganglion (n = 16). In some neurons, pressure application of D-Asp elicited an action

RESULTS

Electrophysiological responses of Aplysia neurons to D-Asp. D-Asp-induced currents were present in 54.1% of cultured BSC neurons (n = 37), 28.6% of non-BSC buccal neurons (n = 7), 18.8% of abdominal ganglion bag cell neurons (n = 16), 40.0% of unidentified cerebral ganglion neurons (n = 25), 45.8% of pleural ventral caudal neurons (n = 59), and 31.3% of unidentified neurons of the pleural ganglion (n = 16). In some neurons, pressure application of D-Asp elicited an action
potential under current-clamp conditions (Fig. 1) that corresponded to an inward current near the resting potential under voltage clamp (Fig. 1, inset), whereas D-Asp elicited only subthreshold depolarizations in others (data not shown). Pressure application of ASW was without effect on BSC neurons (data not shown), indicating excitatory responses were due to the presence of D-Asp.

Under voltage-clamp conditions, some BSC neurons responded to both D-Asp and L-Glu in alternate applications of these agonists. Whole cell currents induced by D-Asp and L-Glu were inward at negative voltages, e.g., −60 and −30 mV, and outward at depolarized voltages, e.g., 60 mV (Fig. 2, A and B). D-Asp- and L-Glu-activated currents possessed a similar I-V relationship. L-Glu-activated currents reversed at −0.4 ± 9.3 mV (n = 7), whereas D-Asp-activated currents reversed at 7.7 ± 9.0 mV (n = 37) in ASW medium and KCl intracellular solution. There was no significant difference in reversal potential between D-Asp- and L-Glu-activated currents; however, currents induced by D-Asp displayed a slight outward rectification compared with those induced by L-Glu (Fig. 2, A and B). In 71 cells in which both agonists were tested, 18 (25.4%) responded to both L-Glu and D-Asp, whereas 35 (49.3%) responded only to L-Glu and 18 (25.4%) responded only to D-Asp, which was significantly different from our expectation (χ² goodness of fit, P < 0.05). Currents activated by L-Glu also displayed a slower time course of activation and inactivation compared with those activated by D-Asp (Fig. 3, A and B; Table 1). Once pressure-applied agonist reached the cell membrane, evidenced by an inflection of the current baseline, D-Asp currents reached peak activation significantly faster than L-Glu currents (P < 0.05, 2-sample t-test). To determine whether duration of agonist exposure affected maximum current or current decay, we compared spontaneous and steady-state current decay. Both spontaneous inactivation in response to a brief, 5-ms pulse of agonist and steady-state inactivation in response to 3-s pulses of agonist were also significantly faster.

Fig. 1. Action potential in a buccal S cluster (BSC) neuron evoked by pressure application of D-Asp (1 mM; 100 ms). Membrane potential (V_m) = −35 mV. Inset: D-Asp-activated whole cell current in the same BSC cell at V_m = −60 mV.

Fig. 2. BSC whole cell currents in response to pressure application of D-Asp and L-Glu. A: D-Asp-evoked currents in a single BSC cell at different holding potentials (1 mM; 100 ms as indicated by horizontal bar). Graph indicates average (±SD) current-voltage (I-V) relationship (n = 55). B: L-Glu-evoked currents in a BSC cell at different holding potentials (1 mM; 100 ms as indicated by horizontal bar). Graph indicates average (±SD) I-V relationship (n = 8).
for d-Asp than for l-Glu (P < 0.05, 2-sample t-test), but spontaneous and steady-state inactivation times for d-Asp were not different, suggesting d-Asp currents rapidly desensitize.

Time constants for activation and inactivation reflected the differences in the kinetics of d-Asp- and l-Glu-evoked currents. The fast and slow activation time constants, \( \tau_a \) and \( \tau_s \), of d-Asp currents were significantly shorter than those for l-Glu (P < 0.05 for \( \tau_a \) and P < 0.01 for \( \tau_s \), 2-sample t-test).

An important distinction between currents elicited by d-Asp and those elicited by l-Glu, in addition to the greater vulnerability of d-Asp currents to desensitization, was their recovery from desensitization. Whereas d-Asp-activated currents rapidly desensitized during repeated applications of agonist, l-Glu-activated currents recovered from apparent desensitization promptly and potentiated over the same time course that produced desensitization in d-Asp currents (Fig. 3C). d-Asp currents recovered from desensitization and returned to maximal amplitude at interpulse intervals of \( \sim 40 \) s (Fig. 4A).

Half-time potential (\( T_{1/2} \)) for recovery was 13 s, whereas \( T_{1/2} \) for recovery of full-amplitude l-Glu currents was <500 ms (data not shown).

Dose-response data were obtained using concentrations of d-Asp between 3 \( \mu \)M and 10 mM (Fig. 4B). Currents were half-maximal at a concentration of \( \sim 42 \) \( \mu \)M d-Asp. The Hill slope coefficient for the fitted curve was 0.67. Current amplitude was maximal at \( \sim 1 \) mM d-Asp.

Ion permeability of d-Asp-activated channels. Each of the major ions in solution was systematically replaced with a larger, theoretically impermeant ion to determine which ions contributed to whole cell d-Asp-induced currents. Significant shifts in the reversal potential under these conditions indicate channel permeation for that ion. Figures 5 and 6 summarize these results.

Na\(^+\) in the ECS was replaced with NMDG, which resulted in a significant negative shift in reversal potential of 47 mV (P < 0.01, Student’s paired t-test; \( n = 7 \)), indicating that Na\(^+\)

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**Table 1. Activation and inactivation times and time constants for pressure-applied d-Asp and l-Glu**

<table>
<thead>
<tr>
<th></th>
<th>Activation (100 ms agonist)</th>
<th>Spontaneous Inactivation (5 ms agonist)</th>
<th>Steady-State Inactivation (3 s agonist)</th>
<th>Inactivation (100 ms agonist)</th>
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<tr>
<td></td>
<td>Time, ms ( \tau_a ) ( \tau_s )</td>
<td>Time, ms ( \tau_i ) ( \tau_s )</td>
<td>Time, ms ( \tau_i ) ( \tau_s )</td>
<td>Time, ms ( \tau_i ) ( \tau_s )</td>
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<tr>
<td>d-Asp</td>
<td>67.9 ± 28.8* (22) 2.9 ± 0.9* (5)</td>
<td>12.7 ± 9.9* (5)</td>
<td>401 ± 223* (16) 497 ± 314* (12)</td>
<td>7.5 ± 4.2* (5) 80.4 ± 46.6* (5)</td>
</tr>
<tr>
<td>l-Glu</td>
<td>121 ± 74.7 (21) 6.2 ± 2.5 (5)</td>
<td>38.1 ± 12.3 (5)</td>
<td>657 ± 462 (15) 595 ± 399 (6)</td>
<td>21.8 ± 11.6 (5) 171 ± 49.4 (5)</td>
</tr>
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Values are means ± SD (no. of neurons is given in parentheses). *P < 0.05, d-Asp vs. l-Glu for the indicated measure.

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was permeant (Fig. 5A). K⁺ in the intracellular solution was also replaced with NMDG and caused a positive shift in reversal potential of 34 mV (Fig. 6A; \( \text{P} < 0.01 \), Student’s paired \( t \)-test; \( n = 7 \)), indicating that K⁺ was permeant.

External Ca²⁺ was replaced in the ECS with NMDG, in addition to the addition of 1 mM EGTA to the ECS to chelate any residual Ca²⁺ (Fig. 5C). Replacing Ca²⁺ did not result in a shift in reversal potential from currents in Ca²⁺-containing ASW (\( n = 12 \)). Because this apparent absence of a reversal potential shift may have been due to the small amount of Ca²⁺ being excluded (ASW contains 10 mM), Ca²⁺ permeability was further investigated by replacing external Mg²⁺ with additional Ca²⁺. Addition of 55 mM extra Ca²⁺ did not cause a shift in reversal potential (Fig. 5D; \( n = 6 \)). The absence of a shift when Ca²⁺ was either excluded or amended in the ECS indicated d-Asp channels were not permeable to Ca²⁺.

Thus d-Asp activated a nonspecific cation channel that excluded Ca²⁺.

Mg²⁺ was also replaced with NMDG to test for voltage-dependent block or permeation through d-Asp channels (Fig. 5B). Replacing external Mg²⁺ with NMDG did not cause a significant shift in reversal potential (\( n = 10 \)). Removing Mg²⁺ caused an increase in current amplitude at voltages ranging from \(-60 \) to \(-15 \) mV (at \(-60 \) mV, amplitude was increased \( 306 \pm 228\% \); at \(-45 \) mV, \( 217 \pm 193\% \); at \(-30 \) mV, \( 189 \pm 159\% \); and at \(-15 \) mV, \( 263 \pm 180\% \); \( P < 0.05 \), Student’s paired \( t \)-test; \( n = 10 \)). Current amplitudes at positive voltages in Mg²⁺-free conditions were not significantly different from those in ASW that contained 55 mM Mg²⁺. Thus d-Asp-activated currents appeared to possess voltage-dependent block by Mg²⁺ similar to that of NMDA receptor channels but lacked the Ca²⁺ permeability of NMDA channels.

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**Fig. 4.** Dependence of whole cell d-Asp current amplitude on frequency of agonist application and on concentration. A: normalized average (±SD) current amplitude in response to a second application of d-Asp in a 2-pulse protocol as a function of the increasing interval between application over 2- to 44-s intervals. Line is fitted to a single-exponential equation (Robert and Howe 2003):

\[
I(t) = I_{\text{max}} - (I_{\text{max}} - I_0) \exp(-t/\tau) \]

(\( n = 8 \); half-time for recovery = 13 s; \( \tau = 18.79 \)). B: average (±SD) dose response for d-Asp. Line is fitted to a Boltzmann equation (Verdoorn and Dingledine 1988):

\[
I/I_{\text{max}} = I_{\text{max}}/[1 + (EC_{50}/[\text{agonist}])^{n}] \]

(\( n = 6 \)). See text for definitions.

**Fig. 5.** Average I-V relationships in d-Asp currents with external cation replacement and higher resolution view of reversal potentials (insets). A: significant reversal potential shift of \(-47 \) mV after replacement of external Na⁺ with N-methyl-D-glucamine (NMDG) (\( P < 0.05 \); \( n = 7 \)). B: absence of shift in reversal potential shift after replacement of external Mg²⁺ with NMDG (\( n = 10 \)). C: absence of shift in reversal potential after replacement of external Ca²⁺ with NMDG (\( n = 12 \)). D: absence of shift in reversal potential after replacement of external Ca²⁺ and Mg²⁺ (\( n = 6 \)). ASW, artificial seawater.
External Cl\textsuperscript{−} was replaced to determine whether this anion contributed to whole cell d-Asp-induced currents (Fig. 6B). NaCl, the major ionic constituent of the ECS, was replaced with Na-gluconate. After correction for a 17-mV junction potential generated by the switch to gluconate, the reversal potential was not significantly different from that in ASW, suggesting that Cl\textsuperscript{−} does not permeate d-Asp-activated channels.

DISCUSSION

The excitatory Na\textsuperscript{+} and K\textsuperscript{+} current activated by d-Asp in BSC neurons had a Hill coefficient for agonist binding near 1, indicating independent binding of d-Asp to its target receptors. These results are consistent with those observed for d-Asp at NMDAR and non-NMDARs expressed in Xenopus oocytes (Verdoorn and Dingledine 1988) and for L-Glu channels at higher agonist concentrations (Patneau and Mayer 1990). Similarities between the whole cell currents induced by d-Asp and L-Glu in BSC cells were their activation ranges, current amplitudes, relatively slow activation and inactivation, and desensitization. These features are most similar to the kinetics of NMDAR channels (Dingledine et al. 1999; Paolletti 2011), the purported site of action of d-Asp (Huang et al. 2005; Kiskin et al. 1990; Olverman et al. 1988). Yet in contrast to L-Glu currents in Aplysia, d-Asp currents had faster kinetics, an outward rectification of the I-V relationship, and a prolonged desensitization on repeated applications of agonist, requiring \( \sim 40 \) s for current amplitude to fully recover. L-Glu currents recovered from desensitization quickly and potentiated as currents were elicited multiple times, characteristic of known L-Glu channels (Heckmann and Dudel 1997; Vicini et al. 1998; Wilding and Huetter 1997).

One of the most conspicuous characteristics distinguishing NMDAR channels from non-NMDAR channels is a high Ca\textsuperscript{2+} permeability (Mayer and Westbrook 1987), not observed presently with d-Asp, and voltage-sensitive Mg\textsuperscript{2+} block (Ascher and Nowak 1988; Dale and Kandel 1993), which we did observe. Since Aplysia has both NMDA-like receptors and AMPA-like receptors free of constitutive block by Mg\textsuperscript{2+} (Li et al. 2005), d-Asp may act at these receptors and/or at novel receptors.

The assertion that d-Asp activates non-L-Glu channels is supported by the observation that approximately one-quarter of BSC cells responded to both L-Glu and d-Asp, whereas another one-quarter responded only to d-Asp and not to L-Glu. Although previous reports have demonstrated separate actions of the L-isoform of aspartate and L-Glu in Aplysia (Yarowsky and Carpenter 1976), d- and L-Asp appear to act independently in BSC neurons (Carlson and Fieber, in press). These data reflect previous results from our laboratory in pleural ganglia (Fieber et al. 2010) but contrast with earlier findings that d-Asp is an alternate agonist of L-Glu channels in vertebrate brain (Kiskin et al. 1990; Olverman et al. 1988). Our results are consistent with findings of Errico et al. (in press) in mouse hippocampal neurons, in which d-Asp activated NMDA receptors but generated excitatory postsynaptic potentials persisting under conditions of NMDAR block. Correspondingly, we demonstrated that d-Asp elicits currents in Aplysia BSC cells that lacked AMPAR and NMDAR currents (Carlson and Fieber, in press). It is possible that the current responses observed in this study represent a mixed population of receptor channels, with d-Asp activating a combination of NMDAR channels and another non-L-Glu channel. This could confound interpretation of results involving d-Asp-induced currents, since some of the channel ion-conducting characteristics observed in this study could be attributed to L-Glu receptor channels. The reported observations, however, were consistent with activation of a population of receptor channels distinct from, at least, L-Glu-activated NMDARs. The finding that d-Asp current responses are modulated by similar mechanisms to AMPAR responses (Carlson and Fieber, in press) suggests that d-Asp activation of a putative d-Asp receptor may substitute for L-Glu activation of AMPARs in some Aplysia neurons. Pharmacological antagonists targeting different L-Glu receptor subtypes may be useful in elucidating the identity of channels activated by both L-Glu and d-Asp and is an area of emphasis. Additional characterization of the endogenous neurotransmitter role of d-Asp will benefit from the use of synaptic preparations, as well as molecular characterization of the receptors activated by d-Asp.

The modulatory actions of d-Asp in previous studies (Brown et al. 2007; Dale and Kandel 1993; Gong et al. 2005) might be explained in terms of high-affinity binding of d-Asp to its receptor, slowing recovery from desensitization (Zhang et al. 2006). d-Asp binding may provide an endogenous mechanism of synaptic modulation if d-Asp binds with high affinity to L-Glu receptors but does not activate them.

Furthermore, the long desensitization time of d-Asp currents may be important in the physiology of the channel, representing an endogenous mechanism for protection from excitotoxicity (Trussel and Fischbach 1989). Cross-activation of d-Asp with L-Glu receptors in mammalian systems may be protective in this respect, and lower levels of free d-Asp in patients with
Alzheimer’s disease would support this hypothesis (D’Aniello et al. 1998).

Our results demonstrate features of the channels activated by d-Asp in the nervous system of Aplysia. d-Asp may have multiple sites of action, similar to l-Glu, and may overlap with l-Glu receptors. Several of our recent results, including some described in this report, however, allude to an undescribed receptor.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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