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Unique ionotropic receptors for D-aspartate are a target for serotonin-induced synaptic plasticity in *Aplysia californica*☆

Stephen L. Carlson [1], Lynne A. Fieber *

University of Miami Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, Miami, FL, USA

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ABSTRACT

The non-L-glutamate (L-Glu) receptor component of D-aspartate (D-Asp) currents in *Aplysia californica* buccal S cluster (BSC) neurons was studied with whole cell voltage clamp to differentiate it from receptors activated by other well-known agonists of the *Aplysia* nervous system and investigate modulatory mechanisms of D-Asp currents associated with synaptic plasticity. Acetylcholine (ACh) and serotonin (5-HT) activated whole cell excitatory currents with similar current voltage relationships to D-Asp. These currents, however, were pharmacologically distinct from D-Asp. ACh currents were blocked by hexamethonium (C6) and tubocurarine (d-TC), while D-Asp currents were unaffected. 5-HT currents were blocked by granisetron and methysergide (MES), while D-Asp currents were unaffected. Conversely, while (2S,3R)-1-(Phenanthren-2-carbonyl) piperazine-2,3-dicarboxylic acid(PPDA) blocked D-Asp currents, it had no effect on ACh or 5-HT currents. Comparison of the charge area described by currents induced by ACh or 5-HT separately from, or with, D-Asp suggests activation of distinct receptors by all 3 agonists. Charge area comparisons with L-Glu, however, suggested some overlap between L-Glu and D-Asp receptors. Ten minute exposure to 5-HT induced facilitation of D-Asp-evoked responses in BSC neurons. This effect was mimicked by phorbol ester, suggesting that protein kinase C (PKC) was involved.

1. Introduction

Molluscs use a variety of small molecules for excitatory synaptic transmission. Among the receptors activated by small molecule neurotransmitters are the cys-family of ligand-gated ion channels. This family includes nicotinic acetylcholine (nACh) and 5-HT_3 receptors. Receptors for both ACh (Kehoe, 1972; Nierop et al., 2005; Nierop et al., 2006) and 5-HT_3 (Walcourt-Ambakederemo and Winlow, 1995; Green et al., 1996; Clemens and Katz, 2001) have been identified in the molluscan nervous system. A complete characterization of D-Asp receptor physiology in the *Aplysia* nervous system includes investigation of the ability of D-Asp to activate these receptors.

D-Asp is present at multiple receptor sites in the *Aplysia* nervous system (Zhao and Liu, 2001). Our related study (Fieber et al., 2010) documented that the buccal S cluster (BSC) neurons have a high preponderance of D-Asp-evoked responses. Receptors activated by D-Asp may overlap with the well-characterized neurotransmitter L-Glu, including N-methyl-D-aspartate receptors (NMDARs) and excitatory amino acid transporters (EAATs), but D-Asp also activates channels independently of L-Glu (Errico et al., 2010; Fieber et al., 2010). Given the unconventional nature of D-isomers as neurotransmitters, with NMDA, D-Ser and D-Asp the sole examples, other ligand-gated channels provide a reasonable starting point for the investigation of the non-L-GluR actions of D-Asp. We hypothesized that the non-L-GluR component of D-Asp whole cell currents may be characterized by activation of cys-family ion channels.

Since the buccal ganglion receives extensive serotonergic innervation (Weiss et al., 1978; Schwartz and Shkolnik, 1981), we further hypothesized that D-Asp currents may be subject to modulation by 5-HT. 5-HT-induced facilitation of sensorimotor synapses (Brunelli et al., 1976) is one of the fundamental processes associated with learning and memory formation in *Aplysia* (reviewed in Glanzman, 2008). At the postsynaptic membrane, sustained exposure to 5-HT activates metabotropic 5-HT receptors inducing a facilitation of L-Glu-evoked responses (Chitwood et al., 2001). This facilitation occurs due to an increase in AMPAR trafficking via exocytosis of vesicular

Abbreviations: Bis, bisindolylmaleimide; BSC, buccal S cluster; C6, hexamethonium; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; D-Asp, D-aspartate; DNQX, 6,7-dinitroquinoxaline-2,3-dione disodium; d-TC, tubocurarine; EAAT, excitatory amino acid transporter; L-Glu, L-glutamate; MES, methysergide; PMA, phorbol 12-myristate 13-acetate; PPDA, (2S,3R)-1-(Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid.

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* Corresponding author at: University of Miami Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, 4600 Rickenbacker Cswy. Miami, FL 33149, USA. Tel.: +1 305 421 4906; fax: +1 305 421 4600.
E-mail address: Lfieber@rsmas.miami.edu (L.A. Fieber).
[1] Current Address: Bowles Center for Alcohol Studies, The University of North Carolina at Chapel Hill, Campus Box 7178, Thurston Bowles Building, Chapel Hill, NC 27599-7178, USA.

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receptor reserves, as well as local, postsynaptic protein synthesis (Li et al., 2005; Villareal et al., 2007). 5-HT appears to produce facilitation via activation of protein kinase C (PKC; Villareal et al., 2009). As D-Asp receptors may be related to ionotropic L-GluRs, we tested D-Asp current modulation by 5-HT. Our results suggest a role for D-Asp specific receptors in synaptic plasticity in *Aplysia*.

2. Materials and methods

2.1. Cell culture

California sea hares, *Aplysia californica*, (~300–500 g; 6–8 months of age and sexually immature) were obtained from the University of Miami NIH National Resource for Aplysia in Miami, FL, USA. Primary cultures of BSC cells were prepared according to the methods in Fieber et al. (2010). Animals were anesthetized for 1 h in a 1:1 mixture of natural seawater from the culture facility and 0.366 M $MgCl_2$. Ganglia were then dissected out and each placed in a 5 ml solution containing 18.75 mg dispase (Boehringer Mannheim), 5 mg hyaluronidase (Sigma), and 1.5 mg collagenase type XI (Sigma) and placed on an elliptical shaker at 114 revolutions/min 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) coated with poly-D-lysine (Sigma, St. Louis, MO, USA). Cells were incubated at 17 °C until used in experiments 24 h later.

2.2. Electrophysiology

Whole cell voltage clamp and current clamp measurements were made using glass patch electrodes pulled from thick-walled 1.5 mm diameter borosilicate glass capillaries using a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA). 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 PC and Digidata 1200 A/D converter using pCLAMP software to record data and issue voltage and current commands (Molecular Devices, Sunnyvale, CA, USA). 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 at a rate of ~0.4 ml/min. 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 attached to a picospritzer powered by N_2 adjustable for pressure and duration (Parker Hannifin, Cleveland, OH, USA). The picospritzer pipette tip 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 picospritzer 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. Unless otherwise noted, in all experiments agonist (D-Asp, L-Asp, NMDA, AMPA, L-Glu, ACh, or 5-HT) was applied via the picospritzer for 100 ms at a concentration of 1 mM, and whole cell current figures are presented at a holding potential of −30 mV.

2.3. Multiple agonist application experiments

For multiple agonist application experiments, an estimate of the net charge passing across the cell membrane in response to activation of D-Asp receptors or receptors activated by another agonist was compared to that produced in response to application of both agonists simultaneously. Whole cell currents in individual cells were elicited by each agonist separately, then both agonists were applied simultaneously using a dual picospritzer pipette mount (Narishige, Japan). The resulting whole cell currents were analyzed using Axograph. Currents were reset to baseline to adjust for leak. The area under the curve from current activation to full inactivation was measured to estimate net charge across the cell membrane. The area described by the current resulting from application of two agonists simultaneously was compared to the area of the current obtained from adding responses to each agonist individually.

2.4. Solutions

Unless otherwise noted, reagents were from Sigma-Aldrich (St. Louis, MO, USA). Extracellular solution (ECS) normally consisted of artificial seawater (ASW) containing (mM) 417 NaCl, 10 KCl, 10 $CaCl_2$ (2 H_2O), 55 $MgCl_2$ (6 H_2O), 15 HEPES-NaOH, pH 7.6 (~physiological pH, unpublished observations). Control intracellular solutions (ICS) contained (mM) 458 KCl, 2.9 $CaCl_2$ (2 H_2O), 2.5 $MgCl_2$ (6 H_2O), 5 Na_2ATP, 10 EGTA, 40 HEPES-KOH, pH 7.4.

Solutions containing D-Asp, L-Asp, NMDA, AMPA, or L-Glu were prepared from frozen 1 M stocks, diluted to 1 mM with the addition of ASW or experimental ECS. ACh and 5-HT solutions were prepared fresh and diluted to working concentrations (1 mM for ACh; 1 mM or 20 μM for 5-HT) in ASW or experimental ECS.

MES was prepared fresh daily at the working concentration of 1 mM in ASW. Other antagonists were diluted in ASW from frozen stocks. Stocks of C6 (1 M), d-TC (100 mM), and granisetron (100 mM; Tocris, St. Louis, MO, USA) were made in water. Stocks of Bis (500 μM; CalBiochem, La Jolla, CA) and phorbol 12-myristate 13-acetate (PMA;

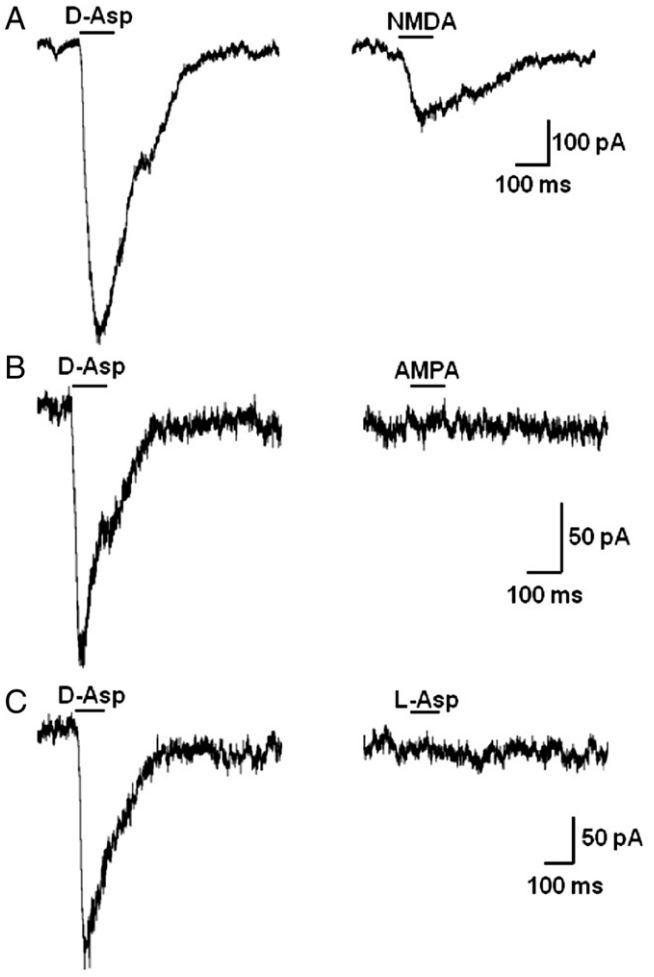

Fig. 1. BSC whole cell current responses induced by D-Asp, specific L-Glu receptor agonists, and L-Asp (1 mM; 100 ms). A. Whole cell currents in a single cell induced by D-Asp (left) and NMDA (right). B. Whole cell currents in a single cell induced by D-Asp (left) and AMPA (right). C. Whole cell currents in a single cell induced by D-Asp (left) and L-Asp (right).