Co-Induction of LTP and LTD and Its Regulation by Protein Kinases and Phosphatases

Kathryn B. Grey and Brian D. Burrell
Neuroscience Group, Division of Basic Biomedical Science, Sanford School of Medicine, University of South Dakota, Vermillion, South Dakota

Submitted 17 December 2009; accepted in final form 22 March 2010

Grey KB, Burrell BD. Co-induction of LTP and LTD and its regulation by protein kinases and phosphatases. J Neurophysiol 103: 2737–2746, 2010. First published March 24, 2010; doi:10.1152/jn.01112.2009. The cellular properties of long-term potentiation (LTP) following pairing of pre- and postsynaptic activity were examined at a known glutamatergic synapse in the leech, specifically between the pressure (P) mecha nosensory and anterior pagoda (AP) neurons. Stimulation of the presynaptic P cell (25 Hz) concurrent with a 2 nA depolarization of the postsynaptic AP cell significantly potentiated the P-to-AP excitatory postsynaptic potential (EPSP) in an N-methyl-D-aspartate receptor (NMDAR)-dependent manner based on inhibitory effects of the NMDAR antagonist MK801 and inhibition of the NMDAR glycine binding site by 7-chlorokynurenic acid. LTP was blocked by injection of bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA) into the postsynaptic (AP) cell, indicating a requirement for postsynaptic elevation of intracellular Ca2+. Autocamtide-2-related inhibitory peptide (AIP), a specific inhibitor of Ca2+/calmodulin-dependent kinase II (CaMKII), and Rp-cAMP, an inhibitor of protein kinase A (PKA), also blocked pairing-induced potentiation, indicating a requirement for activation of CaMKII and PKA. Interestingly, application of AIP during pairing resulted in significantly depressed synaptic transmission. Co-application of AIP with the protein phosphatase inhibitor okadaic acid restored synaptic transmission to baseline levels, suggesting an interaction between CaMKII and protein phosphatases during induction of activity-dependent synaptic plasticity. When postsynaptic activity preceded presynaptic activity, NMDAR-dependent long-term depression (LTD) was observed that was blocked by okadaic acid. Postsynaptic injection of botulinum toxin blocked P-to-AP potentiation while postsynaptic injection of pep2-SVKI, an inhibitor of AMPA receptor endocytosis, inhibited LTD, supporting the hypothesis that glutamate receptor trafficking contributes to both LTP and LTD at the P-to-AP synapse in the leech.

INTRODUCTION

N-methyl-D-aspartate-receptor (NMDAR)-dependent long-term potentiation (LTP) and long-term depression (LTD) have been extensively studied in the mammalian brain as a result of the central role that LTP and LTD play in modifying neural circuits in the context of neural development, sensory processing and learning and memory (Feldman 2009; Massey and Bashir 2007). NMDARs are also present in a wide range of invertebrates including Aplysia, the honey bee, and the leech (Brockie et al. 2001; Grey et al. 2009; Ha et al. 2006; Zannat et al. 2006), and NMDAR-dependent LTP and LTD have been reported in the mollusk Aplysia and the leech Hirudo (Burrell and Sahley 2004; Grey and Burrell 2008; Grey et al. 2009; Li and Burrell 2009; Lin and Glanzman 1994; Murphy and Glanzman 1997, 1999). In vertebrates, many forms of LTP and LTD require activation of NMDARs and increases in postsynaptic Ca2+. In the case of LTP, this increase in Ca2+ activates protein kinase A (PKA) and Ca2+/calmodulin-dependent kinase II (CaMKII), kinases that are thought to stimulate the insertion of additional of glutamate receptors to the postsynaptic surface (Kessels and Malinow 2009; Nayak et al. 1998). In the case of LTD, increases in Ca2+ activate protein phosphatases in hippocampal culture, which ultimately results in the removal of glutamate receptors from the postsynaptic surface (Beattie et al. 2000).

Evidence from Aplysia indicate that LTP in invertebrates also depends on increases in postsynaptic Ca2+ (Lin and Glanzman 1996; Murphy and Glanzman 1996), but it is not known whether invertebrate LTP and LTD utilizes the same cellular signaling processes, e.g., Ca2+/calmodulin kinase or protein phosphatases, as in vertebrates. Understanding the mechanisms that support NMDAR-dependent synaptic plasticity in invertebrates is important given the utility of these animals (especially in regards to their well characterized nervous systems and/or ease of genetic manipulation) in studying fundamental processes in nervous system function, including learning and memory. The leech provides a useful model system for examining the biochemical pathways that mediate pairing-induced synaptic potentiation. The leech possesses a distributed CNS with each ganglion containing ~400 neurons, many of which can be readily identified, making it possible to test synaptic transmission between that same pair of cells across multiple preparations. In addition, removing individual ganglia for electrophysiological recordings preserves all the synaptic connections between neurons, an advantage not seen in slice preparations used in many vertebrate brain studies.

In this study, activity-dependent plasticity is examined at the synapse formed by the pressure-sensitive (P) neuron onto the anterior pagoda (AP) neuron. This P-to-AP synapse is glutamatergic (Wessel et al. 1999) and has both mono- and polysynaptic components (Gu 1991). In addition, NR1, the obligate NMDA subunit, transcript is present in the postsynaptic (AP) neuron (Grey et al. 2009). Previously, NMDAR-dependent potentiation of the P-to-AP synapse was observed following paired activation of the pre- and postsynaptic neurons (Grey et al. 2009) or following forskolin application (“chemical LTP”) (Grey and Burrell 2008). Here the intracellular signaling mechanisms mediating pairing-dependent synaptic plasticity are examined in more detail. LTP was observed when pre- and postsynaptic activity coincided and this potentiation required postsynaptic increases in intracellular Ca2+ and activation of PKA and CaMKII. LTD was observed when postsynaptic...
activity preceded presynaptic activity and required activation of protein phosphatases. Interestingly, when protein kinase activity was blocked during LTP induction, LTP was prevented and LTD was observed. This “uncovered” form of LTD could itself be blocked by application of the protein phosphatase inhibitor okadaic acid, indicating that LTP-inducing protocols can initiate both LTP and LTD signal cascades that interact with each other.

**METHODS**

Leeches, *Hirudo verbana* (Siddall et al. 2007), weighing 3 g were obtained from a commercial supplier (Leeches USA, Westbury, NY, or Niagara Medicinal Leeches, Niagara Falls, Ontario, Canada) and kept in pond water [0.52 g/l H2O *Hirudo* salt (Leeches USA)] at 15°C, under a 12 h light/dark cycle. Individual ganglia were dissected and placed in a recording chamber (1 ml) with constant superfusion (~1 ml/min). Dissections and recordings were carried out in leech saline containing (mM): 115 NaCl, 4 KCl, 1.8 CaCl2, 1 MgCl2, and 10 HEPES. Dual intracellular recordings were made by impaling individual neurons with a glass microelectrode using a micropositioner (Model 1480; Siskiyou, Grants Pass, OR). Electrodes were pulled from borosilicate capillary tubing (1.0 mm OD, 0.75 mm ID, FHC, Bowdoinham, ME) to a resistance of 25–35 MΩ and filled with 3 M potassium acetate. Signals were amplified with a bridge amplifier (BA-1S; NPI, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for viewing and subsequent analysis (Axoscope; Molecular Devices, Sunnyvale, CA). Individual neurons were identified based on their position, size, and action potential shape. Current pulses were delivered to individual neurons using a programmable stimulator (MultiChannel Systems STG 1004). Excitatory postsynaptic potentials (EPSPs) in the AP cell were elicited by brief, 1.5 nA, 10 ms current injections into a contralateral P cell. To prevent the initiation of action potentials, the AP neuron was hyperpolarized to the same membrane potential during both the pre- and posttests (~75 mV). Input resistance of the postsynaptic AP cell was measured throughout each experiment by injecting negative currents (0.5 nA, 500 ms). Typically, four to six EPSPs and seven to nine input resistance measurements were averaged per recording.

In all experiments, baseline EPSP amplitude and input resistance measurements were taken in normal saline. NMDAR-dependent synaptic plasticity requires the co-agonist glycine, therefore 1 mM glycine was superfused in the bath during pairing sessions (Burrell and Sahley 2009). Previous studies have demonstrated that no potentiation was observed when only the P or AP cell was activated (Grey et al. 2009). Previous studies have found that both pairing-dependent LTP and forskolin-induced chemical LTP at the P-to-AP synapse were blocked by the competitive NMDAR antagonist 2-amino-5-phosphonopentanoic acid (AP5) (Grey and Burrell 2008; Grey et al. 2009). To confirm that pairing-induced LTP required NMDAR activation, experiments were carried out in the presence of the NMDAR open channel blocker MK801 (40 μM). The amino acids required for MK801 binding are conserved in the leech (Grey et al. 2009), and MK801 has been shown to block currents in *C. elegans* (Brockie et al. 2001). MK801 blocked the potentiation when applied during pairing (pairing + MK801, 108 ± 8%, n = 6; P < 0.05; Fig. 1C) without an effect on baseline synaptic transmission when applied without pairing (MK801 control, 96 ± 8%, n = 9; F(3,27) = 11.598, P < 0.001; Fig. 1C). No change in input resistance was observed between pairing and no pairing synapses (P > 0.05).

Glycine is an obligatory co-agonist for NMDAR function and testing glycine-dependence of P-to-AP LTP would provide further support for NMDAR involvement. In previous experiments at the P-to-S synapse, glycine was required during
induction of LTP (Burrell and Sahley 2004). Therefore to test whether P-to-AP plasticity showed the same glycine dependence, glycine was omitted from the saline superfusion during the P + AP pairing protocol, and this resulted in no observed potentiation (pairing, no glycine, 104 ± 5%, n = 5; P < 0.05; Fig. 1D). In a second set of experiments, the pairing protocol (with 1 μM glycine) was administered in the presence of 7-Cl-KYNA (20 μM), an inhibitor of the NMDAR glycine-binding site. 7-Cl-KYNA blocked pairing-induced potentiation (pairing + 7-CL KYNA, 97 ± 9%; n = 8; P < 0.05) but did not affect basal synaptic transmission when 7-Cl-KYNA alone (7-Cl-KYNA control) did not significantly affect baseline synaptic transmission. Asterisks indicates statistically significant difference relative to the pairing group.

**Pairing-induced potentiation requires an increase in postsynaptic intracellular Ca^{2+}**

In both mammals and the marine mollusc *Aplysia*, increases in postsynaptic Ca^{2+} are necessary for the expression of LTP (Lynch et al. 1983; Murphy and Glanzman 1996) presumably due to Ca^{2+} influx through NMDARs. To determine whether pairing-induced potentiation requires postsynaptic increases in intracellular Ca^{2+} in the leech, the Ca^{2+} chelator BAPTA (1 mM) was iontophoretically injected into the AP (postsynaptic) cell for 5 min prior to the administration of the pairing protocol. Previous studies indicate that BAPTA reaches synaptic regions of leech neurons within this period of time (Grey and Burrell 2008). Injection of BAPTA into the postsynaptic AP cell before pairing blocked LTP (AP BAPTA + pairing, 85 ± 9%, n = 5; P < 0.05; Fig. 2A), indicating that this potentiation depends on an increase in postsynaptic intracellular Ca^{2+}. Injection of BAPTA into the postsynaptic cell alone did not alter baseline synaptic transmission (AP BAPTA control, 93 ± 8%; n = 5; P < 0.05). There was no significant change in input resistance for any of the BAPTA groups (P > 0.05).

The role of presynaptic Ca^{2+} during pairing-induced LTP was also examined. Another Ca^{2+} chelator, EGTA, was used to assess Ca^{2+} increases in the P cell because this chelator has slower kinetics than BAPTA (Nevian and Sakmann 2006) and therefore would be less likely to disrupt synaptic transmission. Nevertheless, presynaptic EGTA did substantially reduce basal P-to-AP synaptic transmission, even at concentrations as low as 1 μM (Fig. 2B; P-to-AP EPSP tested following 5 min EGTA injection), consistent with observations by Ivanov and Calabr-
FIG. 2. Role of multiple sources of postsynaptic Ca\(^{2+}\) during pairing-induced LTP. A: effects of postsynaptic bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA) injection on LTP. No potentiation was observed when BAPTA was injected into the postsynaptic (AP) cell before administration of the pairing protocol (AP BAPTA + pairing). Postsynaptic injection of BAPTA alone did not affect baseline synaptic transmission (AP BAPTA control). B: effects of presynaptic EGTA injection on the P-to-AP EPSP. After 5 min of iontophoretically injected EGTA into the presynaptic (P) cell, evoked P-to-AP synaptic transmission decreased, on average, 50% from pre-EGTA treatment levels. Top: representative EPSP traces from a P-to-AP synapse treated with 1 \(\mu M\) EGTA. The posttest (post, gray trace) was conducted 1 h after the pretest (pre, black trace). Bottom: averaged change in EPSP size following presynaptic EGTA treatment for concentrations between 1 \(\mu M\) to 0.5 mM. C: effects of presynaptic EGTA injection on LTP. Iontophoresis of 0.5 mM EGTA into the presynaptic (P) cell before administration of the pairing protocol produced potentiation relative to the EGTA control (EGTA treatment, but no pairing). D: effects of nimodipine of LTP. Nimodipine blocked pairing-induced potentiation (nimodipine + pairing). Methanol, the vehicle control for nimodipine, did not affect pairing-induced potentiation (0.1% vol/vol methanol + pairing), and nimodipine administration alone did not affect baseline synaptic transmission (nimodipine control). E: effects of inhibition of release from intracellular Ca\(^{2+}\) stores on LTP. Inhibition of Ca\(^{2+}\) release from intracellular stores also contributed to pairing-induced LTP, which was blocked by depletion of Ca\(^{2+}\) stores by cyclopiazonic acid (CPA), inhibition of IP₃ receptors by TMB-8, or by inhibition of ryanodine receptors by ryanodine. The vehicle controls, DMSO for CPA and TMB-8 and ethanol for ryanodine, did not affect potentiation. *, statistically significant difference relative to the pairing group.

In addition to NMDARs, other sources of Ca\(^{2+}\) may also contribute to the increase in postsynaptic Ca\(^{2+}\) seen in this pairing-induced potentiation, such as voltage-gated Ca\(^{2+}\) channels (VGCCs) and release of Ca\(^{2+}\) from internal stores, the latter potentially mediated by either IP₃ or ryanodine receptors (Balschun et al. 1999; Kapur et al. 1998; Vargas et al. 2007). To test whether LTP requires VGCC activation, nimodipine (10 \(\mu M\)) was superfused during administration of the pairing protocol. Dihydropyridines, such as nimodipine and nifedipine, have been reported to be effective in invertebrates (Dierkes et al. 2004; Elliot et al. 1993; but see Kleinhaus and Angstadt 1995). Because VGCCs are located both presynaptic and postsynaptically, application of nimodipine could potentially interfere with synaptic transmission during administration of the pairing protocol. To test this possibility, the P-to-AP EPSP was tested directly after 10 min of nimodipine application and was not found to affect the P-to-AP EPSP (100 ± 3%, \(n = 3\), data not shown). Application of nimodipine during pairing blocked potentiation without affecting baseline synaptic transmission or input resistance (nimodipine + pairing, 88 ± 2%, \(n = 5\).
nimodipine control, 93 ± 6%, n = 6; P < 0.05; input resistance P > 0.05; Fig. 2D). Nimodipine was dissolved in methanol, yielding a final methanol concentration of 0.1% vol/vol. As a vehicle control for nimodipine, 0.1% vol/vol methanol in saline was applied during pairing, but was not found to disrupt LTP in this synapse [0.1% MeOH + pairing, 164 ± 11%, n = 11; P < 0.05; F(3,24) = 17.043, P < 0.001; Fig. 2D].

The role of Ca\(^{2+}\) release from intracellular stores during LTP was tested by three different drugs. First, CPA inhibits the Ca\(^{2+}\)-pump, thereby depleting internal Ca\(^{2+}\) stores and has been shown to be effective in both Hirudo and Helix neurons (Beck et al. 2001; Willoughby et al. 2001). Superfusion of CPA (20 μM) during P + AP pairing blocked LTP (CPA + pairing, 106 ± 9%, n = 8; P < 0.05), whereas CPA application in the absence of pairing did not affect baseline synaptic transmission (CPA control, 93 ± 7%, n = 5; P < 0.05; Fig. 2E).

Second, to test the role of IP\(_3\)-receptor mediated store-released Ca\(^{2+}\), the pairing protocol was administered in the presence of TMB-8, which has been shown to inhibit IP\(_3\)-induced Ca\(^{2+}\) release in sea urchin eggs (Clapper and Lee 1985). Application of TMB-8 (100 μM) during P + AP pairing blocked LTP (TMB-8 + pairing, 102 ± 8%, n = 8; TMB-8 control, 98 ± 9%, n = 5; P < 0.05; Fig. 2E). Third, the role of ryanodine receptor (RyR)-mediated release from intracellular Ca\(^{2+}\) stores was also examined. Ryanodine, at micromolar concentrations (Sutko et al. 1997), is a selective inhibitor for RyRs and has been shown to be effective in a variety of invertebrates, including Aplysia, Hirudo, and Drosophila (Geiger and Magoski 2008; Trueta et al. 2004; Xu et al. 2000). Application of ryanodine (50 μM) in conjunction with the pairing protocol blocked LTP [ryanodine + pairing, 102 ± 9%, n = 10; ryanodine control, 93 ± 9%, n = 11; P < 0.05; F(8,68) = 10.898, P < 0.001; Fig. 2E], indicating that RyR-mediated Ca\(^{2+}\) release is necessary for pairing-induced potentiation of the P-to-AP synapse. Neither DMSO (0.2% vol/vol), which was used as a solvent for both CPA and TMB-8, nor ethanol (0.5% vol/vol), the solvent for ryanodine, significantly affected LTP (saline (pairing), 163 ± 12%, n = 15; DMSO + pairing, 153 ± 8%, n = 16; ethanol + pairing, 153 ± 7%, n = 9; both vehicle controls P > 0.05 compared with saline (pairing); Fig. 2E). There was no significant change in input resistance for any of the Ca\(^{2+}\) drug groups (P > 0.05; n = 146 total experiments shown in Fig. 2). These results indicate that IP\(_3\)-receptor and RyR-mediated release of Ca\(^{2+}\) from intracellular stores contribute to pairing-dependent LTP in the leech. It is possible that these processes contribute to postsynaptic increases in intracellular Ca\(^{2+}\), but additional experiments using injectable, membrane-impermeant blockers of these receptors are needed to confirm this conclusion.

**Role of CaMKII and PKA during pairing-induced LTP**

Given the requirement for Ca\(^{2+}\) signaling during P-to-AP LTP, the role of Ca\(^{2+}\)-activated biochemical pathways known to mediate LTP in vertebrates, specifically CaMKII and PKA, was investigated (Blitzer et al. 1998; Miyamoto 2006; Yang et al. 2004; Zheng and Keifer 2009). The specific CaMKII inhibitor, AIP, has been shown to block both vertebrate LTP (Yang et al. 2004) and forskolin-induced chemical LTP in the leech (Grey and Burrell 2008). Application of AIP (0.1 μM) during the pairing protocol blocked pairing-induced potentiation in the P-to-AP synapse (Figs. 3, A and B). Interestingly, AIP treatment during pairing resulted in significant depression of the P-to-AP EPSP (AIP + pairing, 57 ± 5%, n = 6, P < 0.05; Fig. 3B). This was observed only when AIP and pairing were combined and was not the result of nonspecific effects of AIP on P-to-AP synaptic transmission. The AIP + pairing group was significantly different from both the pairing group in normal saline and control groups in which AIP was applied but pairing was omitted [AIP control, 101 ± 5%, n = 7, P < 0.05; F(4,29) = 22.751, P < 0.001; Fig. 3B]. In addition, no changes were observed in input resistance as a result of AIP treatment.
between the Rp-cAMP/H11001 (Rp-cAMP) examined. Co-application of OkA and Rp-cAMP during pairing (Rp-cAMP pairing) blocked LTP and produced significant depression of synaptic transmission (P < 0.005) compared with baseline synaptic transmission (control, 100 ± 10%, n = 14; P < 0.05; F(4,51) = 12.252, P < 0.001; Fig. 3C). No changes in input resistance were observed between drug-treated and control groups (P > 0.05; 93 total experiments for Fig. 3). These results suggest pairing-induced potentiation requires activation of the CaMKII and PKA signaling pathways and that these pathways interact with and likely suppress protein phosphatase activity.

Negative pairing can produce NMDAR-dependent LTD

In all of the experiments described so far, LTP has been induced by the simultaneous activation of the pre- (P cell) and postsynaptic (AP cell) neurons. Because alterations in the temporal order of pre- and postsynaptic stimulation can change the polarity of synaptic plasticity (Bi and Poo 1998), the effects of different intervals between the pre- and postsynaptic stimulation during pairing were examined. Specifically, experiments were conducted in which presynaptic stimulation preceded postsynaptic stimulation by 500 ms, 1 s, and 10 s, and postsynaptic preceded presynaptic stimulation by 500 ms, 1 s, and 10 s (referred to as negative intervals). No significant changes in the P-to-AP EPSP were observed at any of the additional intervals tested except when postsynaptic (AP) stimulation preceded presynaptic (P) stimulation by 1 s, which produced significant depression of synaptic transmission [-1 s, post-before-pre, 58 ± 3%, n = 9; P < 0.05 compared with the no stimulation control (not shown in figure); F(7,52) = 7.255, P < 0.001; Fig. 4A, B]. To investigate whether this form of depression was NMDAR-dependent, MK801 (40 μM) was applied during administration of the −1 s pairing protocol. MK801 blocked −1 s pairing-induced LTD, demonstrating this form of synaptic depression is NMDAR-dependent [MK801 + −1 s ISI, 93 ± 6%, n = 6; P < 0.05; F(3,30) = 10.233, P < 0.001; Fig. 4C]. To assess whether protein phosphatase activation is required for LTD, OkA was applied during −1 s pairing. OkA (1 μM) blocked −1 s pairing-induced LTD (OKA + −1 s ISI, 96 ± 6%, n = 7; P < 0.05), despite significantly depressing baseline synaptic transmission [OkA control, 80 ± 7%, n = 7; F(3,29) = 11.592, P < 0.001; Fig. 4D total number of synapses in Fig. 4 is 89].

Glutamate receptor trafficking

Insertion of AMPA-type glutamate receptors into the postsynaptic membrane is thought to be a critical component of LTP expression (Kessels and Malinow 2009). There are no known antibodies that recognize AMPA receptors in the leech; therefore an alternative approach was employed. Botulinum toxin type B (BTX-B) cleaves SNARE proteins necessary for exocytosis (Montecucco and Schiavo 1995), including those in the leech (Bruns et al. 1997). BTX-B injections inhibit synaptic potentiation, presumably by blocking insertion of glutamate receptors into the postsynaptic membrane, and BTX-B injections have been used to inhibit synaptic potentiation in both vertebrates and invertebrates (Antonov et al. 2007; Chitwood et al. 2001; Frey et al. 2009; Jin and Hawkins 2003; Li et al. 2005). Iontophoresis of BTX-B into the postsynaptic AP-cell
prevented pairing-induced LTP (BTX-B + pairing, 104 ± 6%, n = 6; P < 0.05; Fig. 5), while postsynaptic BTX-B treatment by itself did not affect synaptic transmission [BTX-B control, 109 ± 9%, n = 5; P < 0.05; F(3,23) = 11.37, P < 0.001] or input resistance (P > 0.05). These results are consistent with the hypothesis that pairing-induced potentiation in the glutamatergic P-to-AP synapse requires the postsynaptic insertion of glutamate receptors although the possibility that BTX-B is blocking an alternative postsynaptic exocytotic event necessary for LTP cannot be excluded.

Removal of glutamate receptors from the postsynaptic membrane is thought to underlie LTD (Beattie et al. 2000; Lüscher et al. 1999). To test whether inhibition of glutamate receptor removal would block LTD in the leech, −1 s pairing was conducted in the presence of SVKI, a peptide that inhibits the endocytosis of AMPA receptors by blocking interactions between the GluR2 subunit and scaffolding proteins (Daw et al. 2000). Invertebrate AMPA-type glutamate receptors do contain PDZ-binding domains that are responsible for interactions with scaffolding proteins and the mechanisms involved in glutamate receptor trafficking appear to be conserved between vertebrates and invertebrates (Chang and Rongo 2005; Walker et al. 2006). Furthermore, SVKI inhibited synaptic depression in leech T-to-T synapses (Li and Burrell 2008). Iontophoresis of SVKI (100 µM) into the postsynaptic AP cell during −1 s pairing blocked LTD (SVKI + pairing, 97 ± 8%, n = 5; P < 0.05; Fig. 5) but did not affect basal synaptic transmission [SVKI control, 100 ± 7%, n = 5; P < 0.05; F(3,25) = 12.44, P < 0.001; Fig. 5] or input resistance (P > 0.05; n = 36 total experiments shown in Fig. 5). These results are consistent with the hypothesis that LTD in the P-to-AP synapse requires the removal of glutamate receptors from the postsynaptic membrane.

**DISCUSSION**

Paired activation of pre- and postsynaptic spike trains can elicit NMDAR-dependent, bidirectional plasticity in a leech glutamatergic synapse. The pattern of plasticity in the present experiments is similar to what others have observed following pairing of pre- and postsynaptic trains of action potentials in...
both vertebrates and invertebrates (Butts et al. 2007; Lin and Glanzman 1997). Specifically, pairing of bursts resulted in potentiation only when pre- and poststimulation occurred simultaneously (0 ms ISI); when pre- and postbursts were desynchronized, little or no synaptic plasticity was observed. One difference from these earlier findings is that in the present experiments post-before-pre pairing (that is, AP before P) of 1 s elicits robust synaptic depression.

P-to-AP LTP was blocked by the NMDAR antagonist MK801 and by inhibition of the NMDAR glycine binding site, either through application of 7-Cl-KYNA, which blocks the NMDAR glycine binding site, or by omitting glycine during pairing. These findings confirm earlier reports that LTP in the leech P-to-S and P-to-AP synapses was NMDAR-dependent, based on the inhibitory effect of the NMDAR competitive antagonist AP5 (Burrell and Sahley 2004; Grey et al. 2009). LTP in the leech required postsynaptic increases in intracellular Ca$^{2+}$, given that postsynaptic injection of BAPTA prevented pairing-induced potentiation. The results of the presynaptic (P cell) EGTA injections suggest that increases in presynaptic Ca$^{2+}$ are not required for pairing-induced LTP, consistent with previous experiments in which increases in presynaptic Ca$^{2+}$ were not required for NMDAR-dependent LTP induced by forskolin (Grey and Burrell 2008). In addition to NMDARs, increases in intracellular Ca$^{2+}$ appear to require VGCC activation; release of Ca$^{2+}$ from internal stores, mediated by both ryanodine and IP$_3$ receptors may contribute to LTP induction as well. PKA and CaMKII activity is also required for LTP at this synapse as is the insertion of additional glutamate receptors into the postsynaptic membrane. A schematic of the cellular mediators of burst-pairing LTP and LTD explored here is summarized in Fig. 6.

LTD at the P-to-AP synapse was elicited when postsynaptic activity preceded presynaptic activity by 1 s. In addition, P-to-AP LTD was NMDAR-dependent and required protein phosphatase activation and endocytosis of postsynaptic glutamate receptors. These properties have been observed in LTD at other synapses in the leech CNS, indicating that this form of synaptic depression is not limited to the P-to-AP synapse (Burrell and Sahley 2004; Li and Burrell 2008, 2009). These findings indicate that synapses in the leech have the capacity for bidirectional synaptic plasticity.

The evidence for glutamate receptor trafficking during LTP and LTD in the P-to-AP synapse is, admittedly, indirect given that there are no tools to directly label leech glutamate receptors. The ability of BTX-B to block LTD has been used as evidence for glutamate receptor insertion in both vertebrates and invertebrates (Antonov et al. 2007; Chitwood et al. 2001; Frey et al. 2009; Jin and Hawkins 2003; Li et al. 2005). However, it is also possible that BTX-B prevents potentiation via alternative processes, such as blocking the postsynaptic release of a retrograde transmitter necessary for LTP although no evidence of such a retrograde signaling mechanism has yet been reported. Glutamate receptor removal from the postsynaptic membrane contributes to P-to-AP LTD as iontophoresis of SVKI blocked depression following ~1 s pairing of P and AP activity. SVKI selectively inhibits endocytosis of AMPA receptors, and has been shown to prevent LTD in vertebrate synapses, by disrupting the GluR2 subunit interaction with glutamate receptor interacting protein (GRIP), AMPA receptor binding protein (ABP), and protein interacting with C kinase (PICK1) at the GluR2 PDZ-binding domain. Although it is not known precisely what AMPA receptor subtype is trafficked in these leech synapses, the SVKI target sequence is found in invertebrate glutamate receptors, and the mechanisms involved in glutamate receptor trafficking appear to be largely conserved between vertebrates and invertebrates (Chang and Rongo 2005; Walker et al. 2006). In addition, both SVKI and general inhibitors of endocytosis (dynamin inhibitory peptide and concanavalin A) have been found to inhibit LTD in the synapse between touch mechanosensory neurons in the leech (Li and Burrell 2008).

One of the most interesting findings from these experiments is that blocking a signaling pathway that mediates LTP (CaMKII) unmasked LTD in this synapse. These results address a fundamental issue concerning NMDAR-mediated synaptic plasticity. How are NMDAR/ Ca$^{2+}$ signals able to generate both synaptic potentiation and depression? Recent studies have suggested that this bidirectional plasticity relies on cellular switches that change the balance between potentiating and depressing processes to determine the polarity of synaptic plasticity (Graupner and Brunel 2007; Nishiyama et al. 2000; van Woerden et al. 2009). Specifically, changes in cytosolic Ca$^{2+}$ levels, mediated by NMDARs and intracellular Ca$^{2+}$ stores, are sufficient to control both the strength and direction of synaptic plasticity (Bi and Poo 1998). These different cytosolic Ca$^{2+}$ levels activate kinases (which contribute to LTP) and phosphatases (which contribute to LTD) to differing degrees and modeling studies indicate that the signaling molecule that is more strongly activated inhibits the less-activated molecule (Graupner and Brunel 2007; Pi and Lisman 2008; Zhabotinski 2000). For example, phosphatases that mediate LTD are more sensitive to Ca$^{2+}$ than kinases that mediate LTP (such as CaMKII), thus lower levels of intracellular Ca$^{2+}$ would preferentially activate protein phosphatases, which would in turn inhibit protein kinases leading to LTD. When intracellular Ca$^{2+}$ levels are sufficiently high, both the kinases that mediate LTP and the phosphatases that mediate LTD are...
presumably activated, yet LTP “wins out” because the kinases that mediate potentiation also actively inhibit the phosphatase/ LTD pathway. Phosphorylation, particularly autophosphorylation, of CaMKII inhibits protein phosphatase activation and has been shown to be critical to initiate LTP (Fukunaga et al. 2000; Giese et al. 1998). Conversely, active protein phosphatases can dephosphorylate CaMKII as well as initiate processes necessary for LTD (Mulkey et al. 1993; Yoshimura et al. 1999). In addition to CaMKII, PKA also inhibits protein phosphatases, specifically protein phosphatase 1, and by this mechanism, PKA is said to “gate” the induction of LTP (Blitzer et al. 1998). The data from both the CaMKII (AIP) and the PKA (Rp-cAMP) experiments presented in this study support this hypothesis. Inhibition of CaMKII or PKA during pairing not only blocked potentiation but also revealed a form of synaptic depression in the P-to-AP connection. This unmasked depression was blocked when the protein phosphatase inhibitor okadaic acid was applied.

The findings from this study have a number of important implications in terms of evolution and phylogenetic relationship of synaptic function and plasticity between vertebrates and invertebrates. It is well established that invertebrate nervous systems possess many of the molecules necessary for activity-dependent synaptic plasticity in vertebrates (e.g., NMDAR subunits, CaMKII, protein phosphatases) (also see Moroz et al. 2006), but there have been relatively few detailed studies of the cellular properties of invertebrate LTP and LTD. Furthermore, bioinformatic studies comparing the complement of synaptic proteins in vertebrates and invertebrates have suggested a reduced capacity for synaptic plasticity in invertebrates (Ryan and Grant 2009; Ryan et al. 2008). The present results demonstrate that, as in vertebrates, invertebrate synapses are capable of bidirectional modification governed by the relative activation of the same compliment of protein kinases and phosphatases that regulate bidirectional synaptic plasticity in vertebrates. These findings indicate that a number of fundamental processes of activity-dependent synaptic plasticity are conserved between invertebrates and are likely to play a functional role in processes of learning and memory, development and sensory processing.

ACKNOWLEDGMENTS

The authors thank Drs. Joyce Keifer, Cliff Summers, Pat Manzerra, and Yi-Fan Li for helpful comments and suggestions during the preparation of this manuscript.

Present address of K. B. Grey, Dept. of Medicine, Div. of Geriatric Medicine, University of California, San Diego, La Jolla, CA 92039-0746.

GRANTS

This work was supported by a subproject (B. Burrell, project director) of the Division of Research Resources Grant P20 RR-015567 (J. Keifer, PI), which is designated as a Center of Biomedical Research Excellence (COBRE).

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


J Neurophysiol • VOL 103 • MAY 2010 • www.jn.org


2746 K. B. GREY AND B. D. BURRELL