Novel Form of LTD Induced by Transient, Partial Inhibition of the Na,K-Pump in Rat Hippocampal CA1 Cells

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Novel form of LTD induced by transient, partial inhibition of the Na,K-pump in rat hippocampal CA1 cells. J Neurophysiol 91: 239–247, 2004; 10.1152/jn.00722.2003. We tested the hypothesis that transient, partial inhibition of the Na,K-pumps could produce lasting effects on synaptic efficacy in brain tissue by applying a low concentration of the ouabain analogue, dihydroouabain (DHO), to hippocampal slices for 15 min and studying the effects on field excitatory postsynaptic potentials (fEPSPs). DHO caused a suppression of fEPSPs during the application period, but this recovered only partially, to ~80% of control levels, after washout lasting as long as 2 h. The lasting suppression had several properties in common with low-frequency stimulation induced long-term depression (LFS-LTD), including an ability to depotentiate long-term potentiated responses. However, DHO-LTD was insensitive to blockade of N-methyl-D-aspartate or mGlu receptors or to inhibitors of protein kinase C or p38 MAP kinase. DHO-LTD did not co-occlude with LFS-LTD and therefore appears to represent a novel form of LTD. Interestingly, DHO-LTD could be prevented by pretreating slices with iberiotoxin, the selective blocker of large, Ca2+-dependent K+ channels (“big K” BK channels), although this did not affect basal fEPSPs. Certain pathological conditions, including hypoxia and ischemia, are associated with a decrease in Na,K-pump activity and hence DHO-LTD may serve as a model for the effects on neuronal function in these conditions.

INTRODUCTION

Long-term depression (LTD) is a form of synaptic plasticity characterized by a long-lasting reduction of synaptic strength (Bear and Abraham 1996; Linden 1994). LTD can be induced by a number of induction protocols, including synaptic and pharmacological stimulation, many of which are dependent on the age of the animal (Kemp et al. 2000). Synaptic LTD is induced by delivery of a low-frequency tetanic stimulation (LFS). Chemical LTD can be induced by brief application of agonists of N-methyl-D-aspartate (NMDA) (Kamal et al. 1999; Lee et al. 1998) or metabotropic glutamate (mGlu) receptors (Camodeca et al. 1999; Fitzjohn et al. 1999) in the absence of synaptic stimulation. The mGluR-dependent form of LTD can also be induced in CA1 of hippocampus by LFS (Oliet et al. 1997) or a paired-pulse stimulus train (Huber et al. 2000; Kemp et al. 2000). There is a similar diversity in LTD expression mechanisms, which can involve protein synthesis (Zhu et al. 2002), protein phosphatase activation (Mulkey et al. 1993), or internalization of glutamate receptors (Beattie et al. 2000; Morishita et al. 2001; Snyder et al. 2001). LTD involves prolonged stimulation in vitro, and yet it is not clear under what circumstances this would occur in vivo. Prolonged release of agonist and activation of intracellular second-messenger systems does occur under pathophysiological conditions, so the possibility of LTD induction under pathophysiological conditions is an important issue.

One consequence of brain injuries that cause hypoxia or anoxia is a reduction in the activity of Na,K-ATPases (the Na,K-pump) (Haddad and Jiang 1993; Lees 1991; McNamara 1994). Inasmuch as such injuries can cause memory loss and other cognitive disturbances, it is of interest to inquire into the synaptic mechanisms that might be responsible. Ross and Soltész found that early traumatic injury (2000) or high-frequency stimulation (2001) altered the excitability of dentate gyrus interneurons by causing a reduction in Na,K-pump activity. Partial inhibition of Na,K-pump activity by the low-affinity ouabain analogue, dihydroouabain (DHO) increases neuronal excitability (McCann and Alger 1987) but in addition suppresses fEPSPs and EPSPs (Vaillend et al. 2002). Importantly, we found that the low concentrations of DHO used did not significantly affect intrinsic cell properties or extracellular [K+] because they only affect the ouabain-sensitive, α3 and α3, isoforms of the Na-pump (Berrebi-Bertrand et al. 1990; Sweadner 1989) that are not primarily responsible for the “housekeeping” functions of the cells. Hence the suppression of fEPSPs is likely to reflect factors specific to the process of synaptic transmission. The increased excitability represented an increase in EPSP-spike coupling, but we did not investigate the fEPSP depression that began when DHO was present in the bath. The acute fEPSP suppression was accompanied by suppression of fiber volleys, but the lasting suppression was not explored. Although it resembled LTD, no comparison of the two phenomena was made. The purpose of the present work was to determine if the lasting, DHO-induced suppression of excitatory synaptic potentials represents a form of LTD and to begin to investigate the cellular mechanisms involved.

METHODS

Extracellular electrophysiology

Male Sprague-Dawley rats aged 30–60 days (Charles River Laboratories) were deeply sedated with halothane and decapitated in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. The brain was removed, and hippocampi were dissected out...
and cut into 400-μm-thick transverse hippocampal slices on a Vibratome (Series 1000; Technical Products International) as previously described (McCarren and Alger 1987). After recovery for 1 h at room temperature, slices were transferred to a submersion chamber and perfused with saline (29–31°C) at a flow rate of 0.5–1 ml/min (Nicoll and Alger 1981). The saline comprised (in mM): 120 NaCl, 3 KCl, 2 MgSO4, 1 Na2HPO4, 25 NaHCO3, 2.5 CaCl2, and 10 glucose and was saturated with 95% O2-5% CO2 (pH 7.4).

Field potentials were recorded from stratum radiatum and/or s. pyramidale of CA1 with glass microelectrodes broken to a tip diameter of 4–7 μm and filled with 1 M NaCl or with saline (5–15 MΩ). The stimuli were delivered by bipolar stimulating electrodes in s. radiatum and antidromic population spikes were evoked by s. radiatum and alvear stimulation, respectively. Signals were digitized at 10 kHz with an A/D interface (Digidata 1200, Axon Instruments) and analyzed with pCLAMP (Axon Instruments).

Data analysis

The initial slope of the fEPSP (mV/msec) was measured within the first millisecond of the response immediately after the negative peak of the fiber volley. Fiber volley amplitude (mV) was determined from the difference between the initial positive and negative peaks. In the isolated fiber volley experiments, TTX (1 μM) was applied at the end of the experiment for subtraction of the stimulus artifact. Antidromic population spike amplitude (mV) was determined by taking the difference between baseline potential before the stimulus artifact and the negative peak of the response.

To quantify the results, values from 15 consecutive responses in the experimental condition were divided by the mean of 15 consecutive responses in the baseline condition. The magnitude of the change in the response was expressed as the mean percent of control before DHO, high- or low-frequency stimulation (HFS or LFS; means ± SE). Long-term potentiation (LTP) was quantified 35–40 min after HFS; as noted LTD was quantified from 25 to 120 min after DHO application or after LFS. To compare the magnitude of the suppressive effect of DHO in naïve and potentiated slices directly, responses in the depotentiation experiments were also normalized to the mean response 40 min after HFS. Comparisons of mean values were obtained with Student’s paired or unpaired t-test or one- and two-way repeated-measures ANOVAs.

Materials

Stock solutions of DHO (10 mM; Sigma) were prepared in deionized water and bath applied at a final concentration of 20 μM for 15 min in all experiments except where otherwise noted. All other drugs were prepared as concentrated stock solutions and diluted by 1:1000 in saline. Water-based stock solutions included 2-amino-5-phosphonovaleric acid (APV, 50–100 μM; Sigma), LY341495 (100 μM; Tocris), 1,2,3,4-tetrahydro-6-nitro,2,3, dioxobenzof[l]quinoxaline-7-sulfonamide (NBQX, 10 μM; Sigma), tetrodotoxin (TTX, 1 μM; Sigma), ibotenic acid (IBTX, 100 nM; Tocris), and tetraethylammonium (TEA, 2 mM; Sigma). DMSO-based stock solutions included AM-251 (4 μM; Tocris), SB 203580 (25 μM; Tocris), and GF 10920X (bisindolylmaleimide 1; BIS-1, 5 μM; Tocris). The final concentration of DMSO was 0.01% (vol/vol).

Results

Vaillend et al. 2002 reported that a 15-min bath application of DHO suppressed excitatory synaptic responses that did not fully recover after DHO had washed from the slice for ≥20 min. This effect could not obviously be explained by an incomplete removal of DHO because the DHO effects on the Na,K-pump reversed with a time constant of 12 min (cf. Fig. 1B of Vaillend et al. 2002). We now report that fEPSPs do not recover even if the wash from DHO is prolonged to 2 h. For all 17 slices, fEPSP slope was only 81.7 ± 2.1% of control amplitude after 50 min of wash (P < 0.001) and, in 4 slices that were followed longer, fEPSP initial slope was 80.2 ± 2.4% of control after 120 min of wash (Fig. 1A, 2 and 3: P < 0.001). Hence the long-term suppression induced by DHO does not depend on the continued presence of DHO in the tissue, and we refer to it as DHO-LTD. Conventional LTD (LFS-LTD) induced by a low-frequency stimulation of the Schaffer collaterals (1 Hz for 900 pulses) (Dudek and Bear 1992; Mulkey and Malenka 1992) suppressed the fEPSP initial slope to 80.3 ± 2.6% of baseline 50 min after the train (n = 5, P < 0.05, data not shown). Thus the magnitude of DHO-LTD was not significantly different from that of LFS-LTD (DHO-LTD: n = 13 vs. LFS-LTD: n = 5, P > 0.05) and was similar to it in duration.

Simultaneous measurements of fiber volley amplitude (Fig. 1B, open triangles) and fEPSP slope (closed circles), measured 50 and 90 min after the end of the DHO application, showed that fEPSPs were significantly suppressed to 82.8 ± 2.0 and 80.6 ± 2.4% of control, respectively (n = 8 and n = 5, P < 0.05), whereas presynaptic axonal fiber volleys were not different from control amplitudes at these times. The FV-fEPSP coupling ratio (the ratio of the fEPSP initial slope and fiber volley amplitude for a given response) was significantly decreased from 2.9 ± 0.2 to 2.6 ± 0.2 after 50 min of wash (n = 8, P < 0.05) and from 3.2 ± 0.4 to 2.6 ± 0.3 after 90 min of wash (n = 5, P < 0.05), indicating that the ability of a fiber volley to generate a given fEPSP was decreased by DHO. Because of the possibility that fiber volleys were contaminated by overlapping synaptic responses, we also tested the effects of DHO on pharmacologically isolated fiber volleys and simultaneously recorded antidromic population spikes in a separate set of experiments (n = 3; data not shown). Both responses were significantly reduced at 5 min into the wash from DHO (FV: to 86.3 ± 1.6% of control; antidromic PS: to 85.8 ± 2.8% of control), but neither response was significantly different from its control after DHO had been washed from the chamber for 55 min. Thus the long-term suppression of fEPSPs cannot be attributed to persistent impaired fiber conduction. The decrease in the relationship between fiber volley and fEPSP is compatible with either pre- or postsynaptic mechanisms.

To determine if DHO-LTD shared similarities with conventional LTD, we asked if DHO-LTD could depotentiate LTP (Barrionuevo et al. 1980; Fujii et al. 1991; Wagner and Alger 1996). LTP was induced in one of two independent s. radiatum pathways by giving three trains of HFS (100 Hz/1 s) at 20-s intervals to the test pathway (Fig. 2). This protocol produces maximal LTP (e.g., Oliet et al. 1997; Wagner and Alger 1995). The control pathway did not receive HFS. After LTP had been established for ≥40 min (151.0 ± 4.1% of baseline, n = 10), DHO was applied for 15 min and then washed for ≥1 h, after which a second HFS was delivered to the test pathway. Figure 2A shows sample traces of responses from the test and control pathways. LTP of the fEPSP (top left) was induced by HFS in the test pathway but not in the control pathway (bottom left). Traces show the effects of DHO (center column) on these responses and the responses (right) after washing DHO from the chamber. The original control traces are reproduced as a dotted line for comparison. Note the persistent reduction of the fEPSP back to control level (top insets) or below (bottom insets) after
DHO removal. We confirmed that the potentiated responses were depotentiated, and not nonselectively suppressed by DHO, by demonstrating that if an additional bout of HFS was given after wash of DHO for ~45 min (n = 17). The same degree of suppression was maintained in the 4 slices monitored for 120 min after DHO had washed from the slice. Within each group of slices, fEPSP suppression in the wash period was significantly different from control (*, P < 0.001). B: fiber volley amplitude recovered to baseline after DHO was washed from the slices. B1: time course of the fiber volley amplitude (open symbols) and simultaneously measured fEPSP initial slope (closed symbols) from slices in which DHO (20 μM) was applied for 15 min and then washed for 8 min (a: n = 8), 50 min (b: n = 8), and 90 min (c: n = 5). B2: fEPSP slope was significantly decreased after 50-min DHO wash (n = 8, P < 0.05) and after 90-min DHO wash (n = 5, P < 0.05), fiber volley amplitude was not significantly different from control in either group (n = 8 and 5).

DHO removal. We confirmed that the potentiated responses were depotentiated, and not nonselectively suppressed by DHO, by demonstrating that if an additional bout of HFS was given after wash of DHO for >50 min, LTP could be reinstated (Fig. 2B, top). Because the initial potentiating protocol will induce maximal LTP, the ability to reestablish LTP after DHO removal demonstrates a specific reversal of the LTP process (Mulkey and Malenka 1992).

For quantitative comparison of the magnitude of DHO-induced depotentiation with the suppression of naive responses, the fEPSP slope data collected from the unpotentiated pathway at the indicated time points in Fig. 2B were normalized to the LTP values and plotted in C. In the test pathway, after DHO had washed for 5 min and 1 h, the responses were significantly depressed compared with the LTP response but were not significantly different from pre-LTP control levels (60.1 ± 2.8 and 75.6 ± 4.9% of LTP; n = 10, P < 0.05). Responses to stimulation of the unpotentiated control pathway were not altered by the LTP induction protocol, confirming that the test and control pathways were independent. The control pathway responses were suppressed by DHO to essentially the same degree (~20%) as were the responses in naïve slices (see Fig. 1A). These results suggest that DHO causes a long-term suppression of excitatory synaptic responses that resembles LFS-LTD.

DHO-LTD is independent of NMDAR, mGluR or cannabinoid receptor activation

Two forms of LFS-LTD have been identified in the rat hippocampal CA1 region (Oliet et al. 1997). One is mediated
by NMDA receptors and the other is mediated by mGluRs. Inhibition of the Na,K pump in presynaptic nerve terminals could cause an increase in intraterminal [Ca\(^{2+}\)] followed by release of glutamate and activation of these receptors. To determine if DHO-LTD requires activation of NMDA or mGlu receptors, we applied DHO in the presence of 50–100 \(\mu\)M APV or the mGluR antagonist, LY341495 (100 \(\mu\)M). In APV, DHO suppressed fEPSPs to 87.3 \(\pm\) 2.4\% baseline after 50 min of DHO wash \((n = 7, P < 0.05; \text{data not shown})\). In LY341495, DHO suppressed the mean fEPSPs as much \(84.7 \pm 4.7\%\) of baseline, \(n = 3\), although because of the greater variability of the baseline responses in LY34195, this was not statistically significant. In the presence of a combination of APV and LY341495, DHO could depotentiate LTP responses to the same extent \((i.e., \text{to } 62.2 \pm 3.9\%\) of the LTP level; \(n = 3\)\) as in control conditions \(\text{(data not shown)}\). We confirmed that the drug cocktail was effective by showing that it prevented LTP induction by a second bout of HFS. Together these experiments suggest that DHO mediates a long-term suppression of excitatory synaptic responses and reversal of LTP that is largely independent of NMDA or mGlu receptors.

The endocannabinoids anandamide and 2-arachidonylglycerol can reduce glutamate release (Al-Hayani and Davies 2000; Alger 2002; for review) although the mechanism is controversial (Hajos et al. 2001). Endocannabinoids can be released by a variety of endogenous processes (Alger 2002). To test the possibility that endocannabinoids are involved in DHO-LTD, we bathed slices with saline containing the selective CB1 receptor antagonist AM-251 (4 \(\mu\)M) for \(\approx 20\) min prior to the standard DHO application. We also assessed maximal DHO suppression at 5 min after starting the wash, and the point of half-maximal suppression caused by DHO, which occurs at \(\approx 8\) min after beginning DHO application. AM-251 did not prevent DHO-induced suppression at 5 min of wash \((54.1 \pm 0.8\%\) of baseline, \(n = 6, P < 0.001; \text{data not shown})\) or at 25 min of wash \((74.4 \pm 0.2\%\) of baseline, \(n = 6, P < 0.001)\). We did notice that it was not until the 12th minute of DHO application that half-maximal suppression was observed \((81.2 \pm 0.5\%\) of baseline, \(P < 0.001)\). Inasmuch as DHO-induced suppression is usually significant after only 8 min of DHO application, this suggests that AM-251 causes a delay in the onset of the DHO effect, and indeed this is quantitated in Fig. 5A. Thus the initial suppression, although not DHO-LTD, may be mediated in part by cannabinoid receptors.
Inhibitors of p38 map kinase, or protein kinase C, do not prevent induction or expression of DHO-LTD

Thus far the data show that blocking the Na,K-pump did not cause DHO-LTD by liberating some likely extracellular receptor ligands. We next considered the possibility that pump inhibition could cause its effects by activating intracellular effectors downstream of extracellular ligands: e.g., protein kinases. The p38 MAP kinase-signaling pathway has been implicated in the induction and maintenance of mGluR-LTD at both the CA3-CA1 (Bolshakov et al. 2000) and the medial perforant path-dentate gyrus synapse (Rush et al. 2002) in the rat hippocampus. To discover if p38 MAP kinase was involved in DHO-LTD, we preincubated hippocampal slices with the selective p38 MAP kinase inhibitor, SB 203580 (25 μM) for ≥1 h prior to DHO application. We found SB 203580 failed to prevent DHO-induced suppression at 5 min (60.5 ± 1.0% of baseline, n = 7, P < 0.01) or at 25 min of wash from DHO (68.1 ± 0.6% of baseline, n = 7, P < 0.01). Interestingly, SB 203580 delayed the onset of suppression (81.96 ± 1.02 of baseline at 11 min of DHO) as did AM-251, showing that the drug did have an effect (Fig. 5A).

Protein kinase C (PKC) has been implicated in the induction of mGluR-dependent LTD in the rat dentate gyrus, where the PKC-selective inhibitor bisindoylmaleimide I (GF 109 203X; BIS-1) blocked group I mGluR-mediated induction and expression of LTD (Camodeca et al. 1999). Nevertheless, preincubating slices with BIS-1 (5 μM) for ≥1 h before the application of DHO failed to prevent either the induction (46.9 ± 2.6% of baseline, n = 7, P < 0.001, DHO-8 min; 29.4 ± 1.3% of baseline, n = 7, P < 0.001, 5-min wash) or expression of DHO LTD (53.9 ± 0.4% of baseline, n = 7, P < 0.001). It appears that neither of two prominent intracellular cascades implicated in synaptic LTD mediates DHO-LTD.

LFS-LTD and DHO-LTD do not co-occlude

The previous results suggested that, despite phenomenological similarities, LFS-LTD and DHO-LTD might not be caused by the same mechanisms. To obtain direct evidence on this point, we induced first one and then the other form of LTD in a given group of slices. If both forms shared the same mechanisms, then they should co-occlude. We found no evidence for co-occlusion, however (Fig. 3). When DHO-LTD was induced first and allowed to stabilize for 25 min after removing DHO, three subsequent bouts of LFS given at 10-min intervals (each bout consisting of 900 pulses at 1 Hz), produced LFS-LTD of ~25% (as measured from the final DHO-LTD amplitude). That is, DHO-LTD produced at stable depression of ~20% from control amplitudes, and LFS-LTD a further 25% reduction from that level (Fig. 3A, n = 5). Conversely, when saturated LFS-LTD was induced first with three bouts of stimulation, application of DHO for 15 min induced a further stable depression from that level (Fig. 3B, n = 5). In other words, each induction method caused about the same degree of depression whether it was delivered to naïve, or to already depressed, slices. Hence LFS-LTD and DHO-LTD appear to represent distinct phenomena.
IbTX applied only prior to and during DHO application delayed the point of initial suppression of the fEPSP slope during the onset of DHO (99.0 ± 1.0% of baseline at 8 min after beginning DHO application, n = 6) compared with control. The normal DHO-induced suppression was observed soon after the start of the saline wash (35.7 ± 1.7% of baseline, n = 6, P < 0.01) and DHO-LTD was also observed after 25 min of wash (70.2 ± 0.5% of baseline, n = 6, P < 0.01). The data suggest that DHO exerts part of its initial effect by raising the presynaptic [Ca\(^{2+}\)], to a level sufficient to activate the BK channels, which reduces glutamate release, but that this initial effect was not enough to block DHO-LTD.

Comparison of the results in Fig. 4, B and C, suggests that the presence of IbTX during the wash period is necessary to prevent DHO-LTD expression, but IbTX applied only during the wash is insufficient for DHO-LTD prevention. When the application of IbTX coincided with the beginning of the saline wash, it did not reverse the synaptic depression (68.3 ± 0.2% of baseline, n = 3, P < 0.001, 25-min wash, Fig. 4D). We conclude that IbTX had to be present during DHO application and for ≥10–15 min after starting the wash to block the induction or expression of DHO-LTD.

We predicted that application of the broader spectrum K\(^+\) blocker, TEA, would mimic IbTX. TEA, applied at 2 mM, a concentration that blocks BK channels in hippocampal cells (Lancaster and Nicoll 1987) throughout the entire recording period significantly reduced the suppression at 25-min wash (86.3 ± 1.1% of baseline, n = 6, P > 0.1, data not shown) but did not prevent the initial DHO-induced suppression (to 62.2 ± 1.1% of baseline, n = 6, P < 0.001 at 8-min DHO; to 49.8 ± 2.0% of baseline at 5-min wash, P < 0.001).

**Time course of DHO-LTD: a between-groups analysis**

To compare the effects of the various pharmacological manipulations with the control experiments (DHO-only) and each other across the three measured time points, we performed a two-way repeated-measures ANOVA on the group means followed by a Scheffe post hoc analysis. The Scheffe test is the most conservative test for multiple comparisons, maintaining the set alpha value for all comparisons (\(\alpha = 0.01\)). The results of the analysis, which are subdivided by the different time points, are illustrated in Fig. 5.

From Fig. 5A, it can be seen that IbTX, AM-251 and SB 203580 delayed the onset of depression after DHO application. That there was no difference among these three groups suggests the possibility of converging signal pathways. Although we have no direct evidence for this, CB1 receptors have been linked to the activation of the p38 MAP kinase pathway (Bouaboula et al. 1995). BIS-1 slightly decreased the time of
onset of DHO-induced depression. Only IbTX (when it was present throughout) reduced the amount of depression seen at the 5-min wash point (Fig. 5A). DHO-LTD was largely prevented by continuous application of IbTX and TEA (Fig. 5C). Continuous application of IbTX (until 15 min after DHO wash) prevented DHO-LTD in S1: to 95 ± 0.96% of control) and unstimulated (S2: to 95 ± 0.86% of control) pathways. A two-way, repeated-measures ANOVA showed that in IbTX neither S1 nor S2 responses differed from their control values and that both differed from the responses at comparable time points in the absence of IbTX.

**DISCUSSION**

Our data support the hypothesis that Na,K-pump regulation plays a role in synaptic plasticity or pathophysiology. DHO-LTD lasted >2 h and was similar in magnitude to conventional LFS-LTD. DHO also reversed LTP. Inhibition of the Na+/K+ pump by ouabain can increase presynaptic [Na+]i, sufficiently to slow or reverse Na+/Ca2+ exchangers and thereby increase LTD period and for 20 min into the wash (Fig. 6A). We then resumed 0.05-Hz stimulation in the nonstimulated pathway. The fEPSPs in both pathways were significantly different from their respective control values (S1: to 76.6 ± 1.96% of control; S2: to 82.7 ± 1.85% of control) and not different from each other. DHO-LTD did not require periodic action potential activation of BK channels for its induction or expression. We confirmed that the same mechanism of DHO-LTD was operant whether or not the stimulation was given, by applying IbTX to a separate set of slices (n = 5) also studied with the two-pathway protocol (Fig. 6B). Continuous application of IbTX (until 15 min after DHO wash) prevented DHO-LTD in S1: to 95 ± 0.96% of control) and unstimulated (S2: to 95 ± 0.86% of control) pathways. A two-way, repeated-measures ANOVA showed that in IbTX neither S1 nor S2 responses differed from their control values and that both differed from the responses at comparable time points in the absence of IbTX.

**FIG. 5.** Comparisons of fEPSPs at various times and conditions. A: group comparisons at 8 min after DHO onset. The only means that were significantly different from the control group were from the IbTX (1 and 2), AM-251, and SB 203580 experiments. Of these 5, only the BIS-1 mean was significantly different from the rest (*, P < 0.001). See text for details. B: group comparisons after 5 min of wash. In this comparison, only the mean that was different from the control mean as well as from all the others was from the 2nd IbTX experiment (*, P < 0.001). C: group comparisons after 25 min of wash. In this comparison, only the means from the 2nd IbTX and TEA experiments were from the control mean. These two means were not different from each but were significantly different from the other experiments (*, P < 0.001). see text for details.

**FIG. 6.** The occurrence of DHO-LTD is independent of stimulation during DHO application. A: 2 pathways were stimulated alternately at 0.05 Hz by independent stimulating electrodes, S1 and S2, throughout a control period prior to DHO application. The same stimulation was continued via S1 through the DHO application, whereas stimulation to S2 was stopped for the DHO period and for 25 min afterward. When S2 stimulation was started again, the mean of the S2 responses for the last 5 min were reduced to the same degree (to 82 ± 1.85% of control) as S1 responses (to 76.6 ± 1.96% of control). Both levels of depression were highly significantly different from their own control levels (P < 0.001) but not different from each other (P > 0.9). B: IbTX prevents the induction of DHO-LTD whether or not stimulation was given. The same experiment as described in A was repeated on separate slices in the presence of IbTX. Neither S1 nor S2 responses after the removal of DHO were different from their own control levels, or each other (P > 0.8), but both were significantly different from the corresponding responses in the absence of IbTX (P < 0.002). For both groups, n = 5 slices; all comparisons made with ANOVA (see METHODS).
[Ca$^{2+}$], in presynaptic terminals (Blaustein 1993) and release neurotransmitters or neuromodulators. An obvious mechanism for the present experiments was glutamate release. However, unlike the two forms of synaptic LTD in CA1 (Oliet et al. 1997) and depotentiation by LFS-LTD (Wagner and Alger 1996), DHO-induced reversal of LTP appears to be largely independent of NMDA or mGluR activation. Alternatively, endocannabinoids released as a result of synaptic activity can induce LTD (Gerdean et al. 2002), but an antagonist of the CB1 receptor did not prevent DHO-LTD. Finally, there was no evidence of co-occlusion between LFS-LTD and DHO-LTD. We conclude that, although DHO-LTD and LFS-LTD resemble each other phenomenologically, they do not depend on the same cellular mechanisms.

Na,K-pump inhibition can activate signaling pathways downstream of surface receptors. When bound by ouabain, Na$^+$,K$^+$-ATPase triggers Ras-dependent signaling pathways in rat cardiac myocytes and essentially acts as a signal transduction protein (Liu et al. 2000; Mohammadi et al. 2001). We considered p38 MAP kinase and PKC as potential downstream mediators of DHO-LTD but did not affect expression of DHO-LTD by applying inhibitors of these enzymes. BIS-1 did not block the induction of DHO-LTD, in contrast to its effects on mGluR-LTD (Camodeca et al. 1999).

Analysis of the time course of DHO-LTD induction and expression revealed that the CB1 receptor antagonist, AM-251 and the p38 MAP kinase inhibitor, SB 203580, both delayed the onset of fEPSP suppression. Thus endocannabinoids could mediate the initial effects of DHO, although one caveat is that AM-251 is an inverse agonist and can, by sequestering G proteins, affect other G-protein-coupled receptors (Vasquez and Lewis 1999). Inhibition of p38 MAP kinase blocks induction of mGluR-LTD (Bolskakov et al. 2000) and has been linked to CB1 receptor activation (Bouaboula et al. 1995), so it is possible that the similarity of effects of AM-251 and SB 203580 on DHO actions reflect inhibition of the same signaling cascade.

Previous work (McCarren and Alger 1987; Vaillend et al. 2002) showed that low concentrations of DHO had no persistent effects on membrane properties or on extracellular ion concentrations, suggesting that the postsynaptic cells are not strongly affected. However, DHO at low concentrations also does have subtle postsynaptic effects. For instance, it enhances the activation of a voltage-dependent Ca$^{2+}$ conductance (McCarren and Alger 1987) and suppresses NMDA receptor-dependent responses to a greater extent than it does AMPA receptor-dependent responses (Vaillend et al. 2002). In CA1 cells, NMDA-dependent LTP is largely a postsynaptic phenomenon (Malenka and Nicoll 1999), and DHO would therefore have to act postsynaptically to cause depotentiation. Nevertheless, we have no direct evidence for the actual locus of DHO-LTD expression or induction or of DHO-depotentiation. Future work will be required to address these issues.

A possible role for presynaptic increases in [Ca$^{2+}$], in DHO-LTD is suggested by the effects of the BK channel blocker IbTX on DHO-induced suppression of fEPSPs. A combined immunohistochemical and EM study revealed that BK channels are concentrated at glutamatergic nerve terminals in CA1 (Hu et al. 2001). Interestingly Hu et al. found that these channels do not normally take part in repolarizing the terminal action potential. When the presynaptic action potentials are broadened by 100 μM 4-AP, which blocks other K channels, Ca$^{2+}$ enters the terminals, BK-type channels are activated, and transmitter release is reduced. Under these conditions, IbTX enhances glutamate release. Our results are compatible with the speculation of Hu et al. (2001) that BK channels would be activated under hypoxic or ischemic conditions because of an increase in intraterminal [Ca$^{2+}$] and would perhaps have some protective role. Increases in [Ca$^{2+}$], would activate BK channels, speeding repolarization of the terminals and reducing glutamate release. On the other hand, the residual DHO-induced fEPSP suppression in IbTX was similar in magnitude and time and of peak effect to the DHO-induced suppression of the presynaptic fiber volley in control conditions (compare Figs. IB and 4B), which suggests that DHO may differentially affect the various ion channels that modulate fiber conduction and the release process. This idea is compatible with the observations of Hu et al. (2001) that IbTX affected transmitter release but not the shape of the presynaptic fiber volley. It is also compatible with the complex, biphasic time course of fEPSP suppression that we often observed (e.g., Fig. 1). The initial phase would represent suppression of the release mechanism at the nerve terminal, which begins to reverse soon after DHO removal, but which is interrupted by the secondary phase of fEPSP suppression that coincides with the delayed suppression of the fiber volley.

Understanding the prevention of DHO-LTD by continuous application of IbTX is more difficult. When IbTX was present only when DHO was being applied, it did not alter DHO-LTD induction. When IbTX application began as DHO was being washed from the chamber, it had no effect, which showed that persistent activation of BK channels cannot explain DHO-LTD expression. To prevent DHO-LTD, IbTX had been present for an interval that extended from near the end of the DHO treatment period for ~10–15 min into the wash period. Evidently intracellular events initiated during this period by partial inhibition of the Na,K-pumps are largely responsible for DHO-LTD induction. The final interpretation of the data depends on the actual loci of DHO-LTD induction and the relevant IbTX-sensitive channels. Despite their prevalence on presynaptic terminals, such channels do exist on CA1 somata, and therefore a postsynaptic site of DHO-LTD prevention by IbTX is conceivable.

This study highlights the effects of partial Na,K-pump inhibition on neuronal activity and thereby indirectly provides evidence for a physiological or pathophysiological role for endogenous Na,K-pump inhibitors. Although we focused on different neurophysiological endpoints than did Ross and Soltész (2000, 2001), our data are compatible with theirs in suggesting the possibility of long-term synaptic plasticity associated with Na,K-pump dysfunction. Our findings might be relevant for evaluating the role of endogenous ouabain- or DHO-like compounds (Hamlyn et al. 1991; Qazzaz et al. 2000) or other Na,K-pump modulators (Therien et al. 2000) in regulating neuronal synaptic plasticity.

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