It is widely assumed that information storage within the nervous system is rooted in the activity-dependent modulation of synaptic interactions between neurons. Long term potentiation (LTP) is an example of a long lasting, use-dependent increase in synaptic efficacy, which can be elicited with brief pulses of high-frequency stimulation (1). Although the biochemical cascades that subserve LTP remain largely unknown, it is well established that LTP at the Schaffer collateral inputs in area CA1 of the hippocampus requires stimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, postsynaptic calcium flux, and activation of several protein kinase cascades (reviewed in Ref. 2). At present, however, little is known about the signaling components downstream of these protein kinases. It is also unclear whether additional, parallel pathways triggered by NMDA receptor stimulation contribute to LTP induction.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which have classically been studied as regulators of cell proliferation and differentiation. In particular, MAPKs have been identified as primary effectors of growth factor receptor signaling, a cascade that includes activation of Ras, Raf and MEK, the dual-specific protein kinase that activates MAPKs via phosphorylations on threonine and tyrosine residues (reviewed in Ref. 3). Interestingly, two MAPK isoforms, p44 MAPK and p42 MAPK, are widely expressed in post-mitotic neurons in the mammalian nervous system (4, 5), an observation that suggests MAPKs might contribute to the regulation of neuronal function in the adult brain.

Several studies have demonstrated that MAPKs are activated following stimulation of neurotransmitter receptors, protein kinase C (PKC), and neuronal growth factor receptors (6–12). The primary limitation of these studies, however, is that most have been conducted using embryonic neuronal cultures or immortal cell lines such as PC12 cells. As MAPKs are thought to play an important role in development and proliferation, the relevance of such studies to the roles of MAPKs in post-mitotic neurons remains to be established. Thus, at present, little is known of either the regulatory mechanisms or the physiologic role of MAPKs in neurons of the adult nervous system.

We are investigating MAPKs as potential regulators of synaptic plasticity in post-mitotic neurons. In particular, we have studied MAPKs as potential components of the biochemical cascades that subserve LTP induction in area CA1 of the adult hippocampus.

**EXPERIMENTAL PROCEDURES**

Hippocampal Slice Preparation, Pharmacology, and Electrophysiology—400-μm transverse hippocampal slices from male Sprague-Dawley rats, 4–8 weeks old, were prepared with a Vibratome and perfused with a standard saline solution (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM d-glucose, 2 mM CaCl₂, 1 mM MgCl₂, saturated with 95% O₂/5% CO₂) and maintained at 32 °C in a Fine Science Tools interface chamber. Under these conditions, normal excitatory and inhibitory synaptic transmission remain intact. For pharmacological treatments, control and experimental slices from the same hippocampus were maintained in adjacent interface chambers, ensuring that control slices were perfused for the same duration as experimental slices. Electrophysiological recordings from hippocampal slices were conducted as described previously (13). The high frequency stimulation (HFS) paradigm employed in these studies was three sets of 100 Hz stimulation, each lasting 1 s, with an intertrain interval of 20 s, at a stimulus intensity that generated 75% of the maximal pEPSP. For both pharmacological and physiological treatments, slices were removed from the chamber at a predetermined time point and immediately frozen on dry ice. The CA1 subregion between the stimulating and recording electrode was microdissected and stored at −80 °C until assayed.

Western Blot Analyses—The CA1 subregion from individual slices was sonicated briefly in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 4 mM para-nitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 4 μg/ml aprotinin).
Following sonication, the soluble extract was obtained after pelleting the crude membrane fraction by centrifugation at 100,000 × g at 4°C. Protein concentration in the soluble fraction was then measured using a Bradford assay, with bovine serum albumin as the standard. Equivalent amounts of protein for each sample were resolved in 8.5% SDS-polyacrylamide gels, blotted electrothermally to Immobilon, and blocked overnight in TTBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin. Anti-MAPK Western Blotting—Blots were incubated with an anti-serum that recognizes both p44 and p42 MAPK (anti-Erk1-CT, Upstate Biotechnology Inc.), followed by incubation with a horseradish peroxidase-linked goat anti-rabbit IgG, and developed using enhanced chemiluminescence (ECL, Amersham). Anti-phosphotyrosine (APT) Western Blotting—Anti-MAPK blots were stripped for a significant mobility shift (62 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol), reblocked overnight, and incubated sequentially with an anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology Inc.), a biotin-labeled goat antimouse IgG, and a horseradish peroxidase-linked avidin-biotin complex (ABC, Vector), and then developed using ECL. Densitometric analysis of the phosphotyrosine immunoreactivity was conducted using a StudioScan (AGFA) desktop scanner and NIH Image software. Phosphotyrosine immunoreactivity of p44 and p42 MAPK was normalized to protein levels using p44 MAPK immunoreactivity in the anti-MAPK Western blot. Phosphotyrosine immunoreactivities were subjected to a one-way analysis of variance (ANOVA) using the Fisher PLSD test.

RESULTS

Assays for Detecting MAPK Activation—We employed two Western blotting protocols that detect the covalent modifications which underlie MAPK activation. An anti-MAPK Western blot takes advantage of the observation that activated MAPKs display a retarded mobility in SDS-polyacrylamide gel electrophoresis, producing a doublet of immunoreactivity (14). Utilization of an antiserum that detects both p44 MAPK and p42 MAPK (anti-Erk1-CT) allowed us to monitor the mobility of both p44 and p42 MAPK in response to a given treatment as an indicator of activation. The second type of Western blot utilized an antiserum to phosphotyrosine residues in order to detect changes in the phosphotyrosine content of p44 and p42. These two characteristics, mobility shift and increased phosphotyrosine immunoreactivity, have been demonstrated to correlate strongly with MAPK activation (7, 9, 14). As such, they provide an indirect yet reliable index of MAPK activation.

*p42 MAPK, but Not p44 MAPK, Is Activated in Area CA1 following Stimulation of Protein Kinase C—Several lines of evidence demonstrate that PKC is a critical component of the LTP induction cascades in area CA1 of the hippocampus. PKC inhibitors block LTP induction in area CA1 (15), while PKC activators enhance synaptic transmission in area CA1 (16). Furthermore, direct biochemical studies have demonstrated that PKC is activated during the induction and expression of LTP in area CA1 (17, 18). Given these observations, it is interesting to note that MAPKs can be activated in many cell types following stimulation of PKC (7, 12, 19). We therefore determined whether stimulation of PKC leads to MAPK activation in area CA1 of the hippocampus.

Bath application of 10 μM phorbol 12,13-diacetate (PDA), a membrane-permeant activator of PKC, resulted in a doublet of p42 MAPK immunoreactivity, with no change in p44 MAPK immunoreactivity (Fig. 1A, anti-MAPK blot). Reprobing of these samples with APT antiserum revealed an increase in the immunoreactivity of a band that comigrated with the mobility-shifted p42 MAPK, with no change in the immunoreactivity of a band that migrated slightly above native p44 MAPK (i.e. in the presumed position of a mobility-shifted p44 MAPK, Fig. 1A, APT blot). Densitometric analysis revealed that PDA treatment led to a significant increase in the APT immunoreactivity of p42, with no effect on p44 APT immunoreactivity (p42: 599.8 ± 116.2% of control; p44: 107.7 ± 15.0% of control; n = 6; p < 0.001; Fig. 1B). These results indicate that stimulation of PKC leads to p42 MAPK activation in area CA1, and are consistent with the observations of Stratton et al. (12) that phorbol ester application leads to the tyrosine phosphorylation of a ~40-kDa protein in the hippocampal slice.

To determine whether the p44 and p42 APT immunoreactivity bands correspond to p44 and p42 MAPK, respectively, we conducted additional experiments in which we first immunoprecipitated p44 and p42 MAPK from control and PDA-treated area CA1 subregions using the anti-MAPK antibody. We then probed the immunoprecipitates with the anti-phosphotyrosine antiserum. This APT Western blot was then stripped and reprobed with the anti-MAPK antibody. These experiments directly demonstrated that p42 MAPK, but not p44 MAPK, was tyrosine phosphorylated in response to PDA treatment, and that the p42 phosphotyrosine immunoreactivity resided with the mobility-shifted band (data not shown). Together, these data strongly suggest that stimulation of PKC leads to the activation of p42 MAPK, but not p44 MAPK, in area CA1.

*p42 MAPK, but Not p44 MAPK, Is Activated in Area CA1 following NMDA Receptor Stimulation—It is well established that stimulation of the N-methyl-D-aspartate subtype of glutamate receptor is required for most forms of LTP in area CA1 (2, 20). Although stimulation of the NMDA receptor has been shown to lead to MAPK activation in some neuronal culture systems (6), such studies have not been reported on post-mitotic neurons in the adult nervous system. We found that, in area CA1, bath application of 100 μM NMDA for 3 min led to the characteristic mobility shift and tyrosine phosphorylation of p42 MAPK, without affecting p44 MAPK mobility or APT immunoreactivity (Fig. 2A, left panels). The NMDA-induced mobility shift and increased phosphotyrosine immunoreactivity of p42 MAPK was blocked completely by the selective NMDA receptor antagonist, d-2-amino-5-phosphonovalerate (d-APV, 100 μM; Fig. 2A, right panels). Quantitative analysis of p44/p42 MAPK APT immunoreactivity is shown in Fig. 2B. NMDA treatment led to a significant increase in the APT immunoreactivity of p42 MAPK, with no effect on p44 MAPK APT immunoreactivity (p42 MAPK-
Response to LTP-inducing High Frequency Stimulation—receptor stimulation results in the activation of p42 MAPK, but not p44 MAPK, in area CA1. Our pharmacological data directly link two important components of the LTP induction cascades in area CA1 (PKC and NMDA receptors) with p42 MAPK activation. These observations led us to hypothesize that p42 MAPK is activated during the induction of LTP in area CA1. As one described function of MAPKs is the regulation of gene transcription (reviewed in Ref. 21), we chose as our LTP induction protocol a paradigm that reliably elicits a transcription/translation-dependent phase of LTP at Schaffer-collateral inputs into area CA1, a phase known as late-LTP (22, 23). As shown in Fig. 3, the delivery of this induction paradigm (three sets of HFS spaced over a 20-min period) reliably produced a long lasting, robust enhancement of the initial slope of the pEPSP (average increase: 211 ± 12% of control, 60 min following the delivery of the HFS paradigm) (n = 9).

Experiments addressing the role of the cyclic AMP-dependant protein kinase cascade in establishing late-LTP have suggested that kinases involved in mediating changes in gene expression are likely to be rapidly and transiently activated by LTP-inducing stimulation (24, 25). It is also known that PKC, which we hypothesize to be upstream of MAPKs, is activated within 1–2 min following LTP-inducing HFS (18). Given these observations, we chose to analyze MAPK activation 2 min following the delivery of the HFS paradigm.

We first analyzed individual area CA1 subregions from 18 slices that received this LTP-inducing HFS paradigm. For each, physiologic responses were monitored up to the 2-min time point to insure the efficacy of the HFS paradigm (Fig. 4A, closed circles). Note that, to this time point, the responses measured in these slices were indistinguishable from responses in which LTP was followed for one hour (Fig. 3). Healthy slices from the same hippocampus, which were maintained in the recording chamber for the duration of the experiment, were used as control slices. Western blot analyses of each of these area CA1 subregions revealed that HFS led to the characteristic mobility shift and increased phosphotyrosine immunoreactivity of p42 MAPK, with no observable change in p44 MAPK mobility or APT immunoreactivity (Fig. 4B, CTL and HFS). Densitometric analysis of APT immunoreactivity showed a significant increase in p42 MAPK, with no change for p44 MAPK (p42 MAPK: 163.2 ± 8.4% of control; p44 MAPK: 103.3 ± 5.1% of control; n = 18; p < 0.001; Fig. 4C). These data strongly suggest that p42 MAPK, but not p44 MAPK, is activated in response to LTP-inducing HFS.

We next determined whether p42 MAPK is activated when the HFS paradigm is delivered in the presence of the NMDA receptor antagonist APV, a compound known to block the induction of LTP. As shown in Fig. 4A, bath application of 50 μM APV blocks the potentiation due to HFS (open circles, n = 5). For the Western blot analyses, these five samples were compared to area CA1 subregions of slices from the same hippocampus, which were perfused with APV for the duration of the experiment. As seen in Fig. 4B, APV prevents the mobility shift and increased phosphotyrosine immunoreactivity of p42 MAPK (APV and HFS + APV). Densitometric analysis of APT immunoreactivity showed no change for either p42 MAPK or p44 MAPK (relative to respective APV control; p42 MAPK: 107.0 ± 3.1%; p44 MAPK: 105.2 ± 5.2%; n = 5; Fig. 4C). These data demonstrate that stimulation of NMDA receptors is necessary for p42 MAPK activation in response to HFS.

As an additional control, we delivered test stimulation alone (i.e. without the HFS paradigm) for the same duration as in the previous two conditions to determine whether this was sufficient to elicit p42 MAPK activation. Physiologic responses for four samples analyzed are shown in Fig. 4A (closed diamonds). Again, control area CA1 subregions were obtained from slices from the same hippocampus which were perfused for the duration of the recordings. As seen in Fig. 4 (B and C), test stimulation alone had no effect upon mobility or tyrosine phosphorylation of either p44 MAPK or p42 MAPK (APT immunoreactivity relative to control levels; p42 MAPK: 100.5 ± 3.5%; p44 MAPK: 95.5 ± 7.1%; n = 4). These results show that test stimulation alone has no effect upon p42 MAPK or p44 MAPK activation in area CA1.
DISCUSSION

MAPKs are excellent candidates for regulators of synaptic plasticity in post-mitotic neurons of the adult nervous system. First of all, MAPKs are expressed in dendrites and somas of pyramidal neurons of the adult nervous system (5), a localization that suggests a role for MAPK in postsynaptic functions. Second, MAPKs can be activated in neuronal culture systems by several neurotransmitters; such observations identify MAPKs as putative targets of neurotransmitter receptors in developed neurons. Finally, substrates of MAPKs regulate many processes thought to be important in synaptic plasticity. These include second messenger generation (e.g., cytosolic phospholipase A2; Ref. 26), cytoskeletal modulation (e.g., MAP2 protein; Ref. 27), and transcription (e.g., Elk-1 transcription factor; reviewed in Ref. 21).

From these observations, we hypothesized that MAPKs were involved in the regulation of synaptic plasticity in post-mitotic neurons of the adult nervous system. In particular, we have studied MAPKs as potential components of the biochemical cascades that subserve LTP induction in area CA1 of the adult hippocampus. We first observed that p42 MAPK, but not p44 MAPK, is activated following direct stimulation of two required components of the LTP induction cascades, namely PKC and NMDA receptors. Second, we demonstrated that p42 MAPK, but not p44 MAPK, is activated in response to LTP-inducing high frequency stimulation and that this activation requires NMDA receptor stimulation. Our results demonstrate that p42 MAPK can be regulated in an activity-dependent manner in the hippocampus and identify it as a potential component of the LTP induction cascades in area CA1.

It is important to note that the samples analyzed contain soluble extracts from the entire CA1 subregion, a region that contains predominantly pyramidal cells, but also contains Schaffer collateral axons, interneurons and glial cells. Immunohistochemistry studies of area CA1 of the adult hippocampus, however, have localized p42 MAPK to the soma and dendrites of pyramidal neurons, with little to no staining in nonpyramidal cells (5). This observation suggests that the activation of p42 MAPK detected by our assays most likely occurred in pyramidal neurons.

Finally, the physiologic role of p42 MAPK in regulating synaptic transmission in area CA1 remains to be established. Still, several lines of evidence indirectly suggest that MAPK activation is necessary for many forms of potentiation in area CA1. First of all, it has been demonstrated that broad-spectrum inhibitors of protein-tyrosine kinases block the induction of NMDA receptor-dependent LTP in area CA1, the form of LTP we have studied (28). The Ras-dependent MAPK activation pathway is the most likely target of this required protein-tyrosine kinase activation. Second, a recent report has demonstrated that stimulation of neurotrophic receptors induces a robust, long lasting potentiation of synaptic responses (29). Here, again, the Ras-dependent MAPK pathway is the most likely signaling pathway. It will be interesting to determine the signal transduction pathway and downstream effectors of MAPKs in post-mitotic neurons, as well as their role in the physiology of LTP and other forms of synaptic plasticity.

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