

ERK Plays a Regulatory Role in Induction of LTP by Theta Frequency Stimulation and Its Modulation by β -Adrenergic Receptors

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Summary

MAP kinase (ERK) translates cell surface signals into alterations in transcription. We have found that ERK also regulates hippocampal neuronal excitability during 5 Hz stimulation and thereby regulates forms of long-term potentiation (LTP) that do not require macromolecular synthesis. Moreover, ERK-mediated changes in excitability are selectively required for some forms of LTP but not others. ERK is required for the early phase of LTP elicited by brief 5 Hz stimulation, as well as for LTP elicited by more prolonged 5 Hz stimulation when paired with β 1-adrenergic receptor activation. By contrast, ERK plays no role in LTP elicited by a single 1 s 100 Hz train. Consistent with these results, we find that ERK is activated by β -adrenergic receptors in CA1 pyramidal cell somas and dendrites.

Introduction

In nonneuronal cells, p42–44 MAP kinase (ERK1/2) has emerged as a key component in a cascade that couples mitogenic signals at the cell surface to transcription. Recent studies suggest that this cascade is also recruited in neurons for transcription-dependent long-term synaptic plasticity (reviewed by Impey et al., 1999; Orban et al., 1999). At the sensory neuron–motor neuron synapse of the gill withdrawal reflex of *Aplysia*, repeated pulses of serotonin recruit ERK, causing it to translocate to the nucleus where it is required for long-term synaptic facilitation (Martin et al., 1997). Similarly, in mammalian hippocampus, repeated high-frequency tetanization at the Schaffer collateral–CA1 pyramidal cell synapse activates ERK and induces a macromolecular synthesis–

dependent form of long-term potentiation (LTP). Inhibitors of ERK signaling reduce LTP evoked by repeated high-frequency tetani (English and Sweatt, 1996, 1997; Atkins et al., 1998; Impey et al., 1998).

In addition to the roles of ERK in persistent cellular alterations achieved by nuclear signaling, studies in PC12 cells indicate that ERK can be recruited for transient cytoplasmic signaling. While NGF activates ERK in a persistent fashion, leading to its translocation to the nucleus and to the initiation of transcription of genes involved in differentiation, EGF activates ERK only transiently in the cytoplasm where it induces proliferation rather than differentiation (Marshall, 1995). These findings raise two questions. First, is this dual action of ERK general? Second, can ERK also participate in more transient regulation in neurons for short-term neuronal plasticity?

One clue to a possible role for ERK in transient signaling comes from studies of the cyclic AMP (cAMP) pathway. One of the interesting features of the cAMP pathway is that it can recruit ERK activation in neurons (Martin et al., 1997; Impey et al., 1998). It is well established that the cAMP pathway participates in both transient as well as in persistent forms of synaptic plasticity (Huang et al., 1994, 1996; Weisskopf et al., 1994; Blitzer et al., 1995, 1998; Winder et al., 1998). Does protein kinase A (PKA), which recruits ERK for persistent forms of synaptic plasticity, also recruit ERK for more transient forms of plasticity that are independent of macromolecular synthesis?

We have examined this question at the Schaffer collateral–CA1 pyramidal cell synapse. We find that ERK plays a critical role in a form of LTP that is elicited by theta frequency (5 Hz) stimulation, which requires PKA but not macromolecular synthesis. ERK contributes to this form of LTP by regulating neuronal excitability during tetanization. Inhibition of ERK reduces the appearance of “complex spike-like” events during 5 Hz stimulation and blocks the LTP evoked by this stimulation. By contrast, PKA-independent LTP elicited by a single 1 s 100 Hz train does not require ERK. Thus, these data suggest that different patterns of stimulation produce LTP by recruiting different molecular signaling pathways and that the role ERK plays in LTP is dependent upon the type of stimulus used to evoke LTP.

The ability of 5 Hz stimulation to elicit LTP depends on the duration of stimulation, with shorter protocols being more effective than prolonged ones. This dependence on the duration of stimulation is removed by the pretreatment of hippocampal slices with the β -adrenergic receptor (β -AR) agonist isoproterenol (Iso; Thomas et al., 1996). Using knockout mice lacking specific β -AR subtypes, we find that this regulation is mediated by the β 1-AR. Using confocal imaging of hippocampal slices incubated with an antibody specific to activated ERK, we find that β -AR stimulation activates ERK in the dendrites and cell body with a time course appropriate for regulation of cellular excitability during 5 Hz stimulation. Moreover, LTP and complex spike-like responses elicited by pairing Iso and prolonged 5 Hz stimulation

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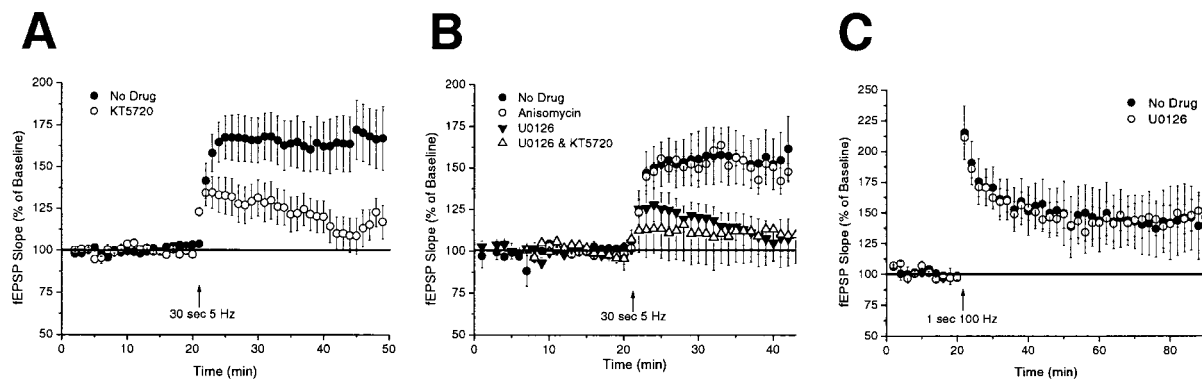


Figure 1. Properties of LTP Elicited by 30 s of 5 Hz Stimulation

(A) Effect of pretreatment of hippocampal slices for 1 hr with 1 μM KT5720 on LTP elicited by a 30 s 5 Hz tetanus ($n = 8$). (B) Effect of pretreatment of hippocampal slices for 30 min with 30 μM anisomycin, for 1 hr with 20 μM U0126, or for 1 hr with U0126 and 1 μM KT5720 on LTP elicited by a 30 s 5 Hz tetanus (vehicle, $n = 6$; anisomycin, $n = 5$; U0126, $n = 8$; U0126 and KT5720, $n = 6$). (C) Effect of pretreatment of hippocampal slices for 1 hr with U0126 on LTP evoked by a single 1 s 100 Hz train.

are reduced by blockers of ERK activity. Thus, these data suggest a novel postsynaptic role for ERK independent of its actions in the nucleus, and establish ERK as a physiologically relevant signaling molecule by which β -ARs and PKA regulate neuronal function.

Results

Brief Theta Frequency Stimulation Induces LTP that Is Dependent on Both PKA and ERK

The theta rhythm (3–12 Hz) of electroencephalograms (EEGs) recorded in the hippocampus is thought to reflect attentive animal tasks (Oddie and Bland, 1998). Brief stimulation in this frequency range is extremely effective in producing LTP. For example, 30 s of 5 Hz stimulation evokes robust LTP that is pathway specific. Twenty minutes after 5 Hz stimulation, the percent increase in the slope of the field excitatory postsynaptic potential (fEPSP) is $176\% \pm 31\%$ in the tetanized pathway and $108\% \pm 4\%$ in the nontetanized pathway ($n = 9$). Moreover, this LTP is predominantly dependent on the NMDA receptor. Twenty minutes after tetanization in 100 μM D,L-AP5 ($n = 4$), the tetanized pathway is only $128\% \pm 13\%$ of baseline. Unlike LTP elicited by a single high-frequency (100 Hz) train, the LTP elicited by brief 5 Hz stimulation is reduced by the PKA inhibitor H89 (Thomas et al., 1996). Consistent with these results, we find that pretreatment of slices with the PKA inhibitor KT5720 (1 μM) similarly reduced LTP elicited with this protocol (Figure 1A).

PKA can have both protein synthesis-dependent and -independent roles in LTP (Huang et al., 1996; Winder et al., 1998). The finding that inhibitors of PKA reduce early components of LTP elicited by brief 5 Hz stimulation suggests that this role of PKA is likely to be independent of macromolecular synthesis. Indeed, this form of LTP is not inhibited by preincubation of hippocampal slices with saturating concentrations of the protein synthesis inhibitor anisomycin (Figure 1B).

Because PKA is required for early components of LTP elicited by 5 Hz stimulation, we determined whether ERK, which can be recruited by PKA, also participates in this component of LTP by pretreating slices for 1 hr

with a membrane-permeable MAP kinase kinase (MEK) inhibitor, U0126 (20 μM ; Favata et al., 1998). This concentration of U0126 completely blocks both basal and stimulus-induced activation of ERK in hippocampal slices, while having no effect on PKA, calcium/calmodulin-dependent protein kinase II (CaMKII), or PKC (Roberston et al., 1999). In slices pretreated with U0126, LTP elicited with brief 5 Hz stimulation returned to baseline within 20 min of the tetanus (Figure 1B). Coadministration of U0126 and KT5720 produced a similar level of inhibition (Figure 1B). These data thus suggest that ERK and PKA are required for LTP elicited by transient 5 Hz stimulation.

ERK Does Not Play a Significant Role in Early LTP Elicited by a Single 100 Hz Train

LTP elicited by multiple 100 Hz trains is dependent on ERK (English and Sweatt, 1997; Impey et al., 1998). We wondered whether ERK plays a general role in all forms of LTP, or whether it is recruited selectively for specific forms of LTP. We therefore examined LTP elicited by a single 1 s 100 Hz train. This form of LTP does not require PKA or macromolecular synthesis (Huang et al., 1996). We found that pretreatment of hippocampal slices with 20 μM U0126 had no effect on LTP elicited by a single train, suggesting that ERK does not play a general role in LTP but rather is recruited selectively for distinct forms (Figure 1C).

LTP Elicited by Prolonged Theta Frequency Stimulation Is Enhanced by Iso

Although brief 5 Hz stimulation elicits robust LTP, prolonged 5 Hz stimulation (3 min) elicits only a transient depression of synaptic transmission that relaxes back to baseline shortly after cessation of 5 Hz stimulation (Figure 2A). However, robust LTP is induced if prolonged 5 Hz stimulation is paired with β -AR activation (Iso, 1 μM ; Figure 2A; Thomas et al., 1996; Katsuki et al., 1997). Although Iso enhances basal synaptic transmission slightly, this enhancement is variable and transient in the absence of tetanic stimulation (Figures 2A and 2B). This is in contrast with other agents that activate cAMP signaling and elicit lasting, macromolecular synthesis-

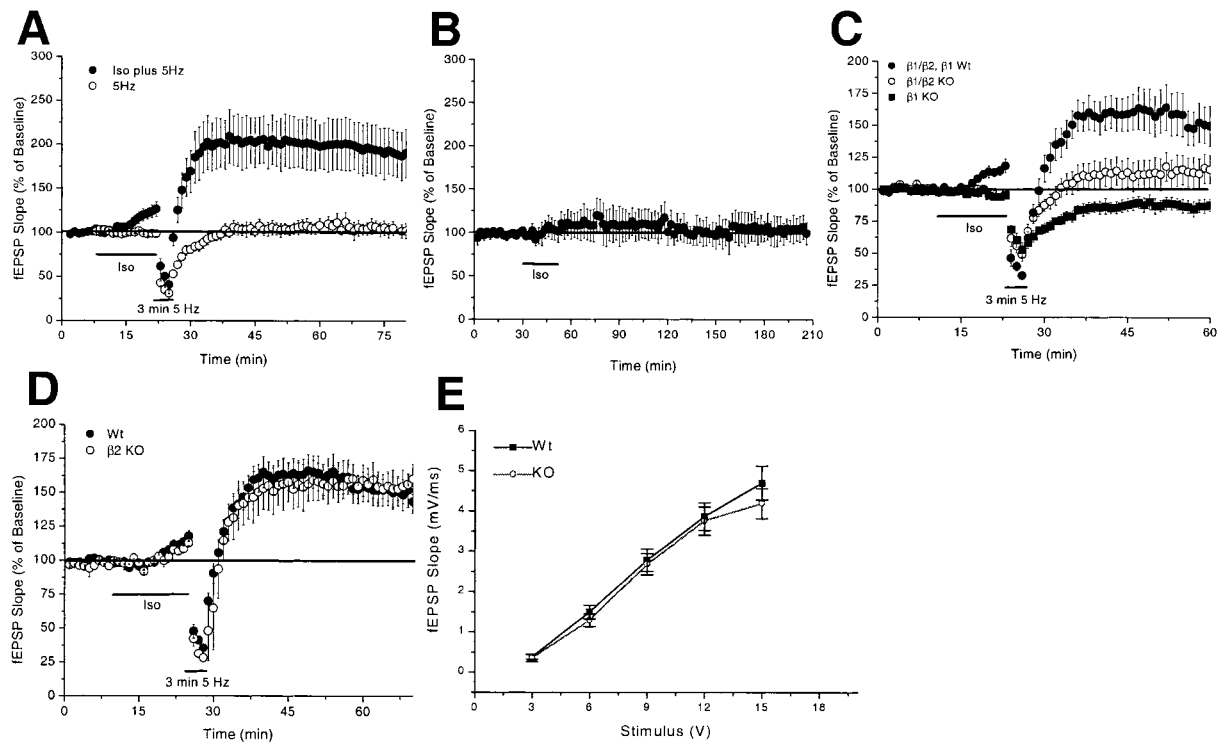


Figure 2. Properties of LTP Elicited by 3 min of 5 Hz Stimulation Coupled with β -AR Activation

(A) Three minutes of 5 Hz stimulation alone elicits a transient depression of transmission that decays back to baseline ($n = 14$). However, pairing this with prior β -AR activation results in the generation of robust LTP ($n = 6$).
 (B) Iso administered (as indicated by the bar in each panel) in the absence of tetanization elicits only a very weak and transient enhancement of synaptic transmission ($n = 4$).
 (C) LTP evoked by pairing Iso and 3 min of 5 Hz stimulation is impaired in hippocampal slices from $\beta 1$ -AR knockout and $\beta 1/\beta 2$ -AR double knockout mice ($\beta 1$, $n = 6$; $\beta 1/\beta 2$, $n = 9$). Wild-type time course represents the data obtained from wild types of both the $\beta 1$ and $\beta 1/\beta 2$ knockout lines ($n = 12$).
 (D) LTP evoked by pairing Iso and 3 min of 5 Hz stimulation is normal in hippocampal slices from $\beta 2$ -AR knockout mice ($n = 3$). There was a trend for the remaining response in slices from double knockouts to be greater than those in $\beta 1$ -AR knockouts, although this trend did not reach statistical significance ($p = 0.17$, Student's t test, 20 min after tetanus). The response to 3 min of 5 Hz stimulation in the absence of Iso was indistinguishable between $\beta 2$ knockouts and FVB controls, suggesting that the absence of the $\beta 2$ -AR gene did not produce a general alteration in the response to 5 Hz stimulation (percent of baseline fEPSP slope 30 min after 5 Hz stimulation: $115\% \pm 9\%$ in knockout, $n = 7$; $115\% \pm 13\%$ in wild type, $n = 6$). It should be noted that the $\beta 2$ -AR knockout mice examined were on an FVB background, whereas the $\beta 1$ -AR knockout mice examined were a cross of C57b6 and 129 strains. Since the $\beta 1/\beta 2$ -AR double knockout created by the mating of these two strains contains a mixture of FVB, C57b6, and 129, this trend may reflect differences in the strains of these animals.
 (E) Input-output plots of stimulus intensity versus fEPSP slope in hippocampal slices from $\beta 1$ - and $\beta 1/\beta 2$ -AR wild-type mice ($n = 23$ slices from 7 mice) versus $\beta 1$ - and $\beta 1/\beta 2$ -AR knockout mice ($n = 20$ slices from 7 mice).

dependent increases in synaptic transmission in area CA1.

Iso-Initiated LTP Requires the $\beta 1$ Subtype of Adrenergic Receptor

The mRNAs for all three subtypes of β -ARs are present in hippocampus (Nicholas et al., 1993; Summers et al., 1995), and β -AR agonists can have a variety of effects on CA1 pyramidal cells. To determine which specific subtype(s) is involved in the regulation of LTP by Iso, we took a genetic approach, using mice with targeted deletions of the $\beta 1$ - or $\beta 2$ -ARs, as well as mice with deletions of both receptors (Rohrer et al., 1996, 1998, 1999; Chruscinski et al., 1999). Both the transient increase in synaptic transmission as well as the facilitation of LTP by Iso ($1 \mu\text{M}$) were substantially reduced in slices from mice lacking either the $\beta 1$ -AR ($p < 0.001$, Student's t test, 20 min after tetanus) or mice lacking both the $\beta 1$ - and $\beta 2$ -ARs ($p < 0.02$, Student's t test, 20 min after

tetanus; Figure 2C) but not in slices from knockout mice lacking the $\beta 2$ -AR (Figure 2D).

The lack of effect of Iso on basal transmission and LTP in hippocampal slices from the $\beta 1$ - and $\beta 1/\beta 2$ -AR knockout mice was not a result of a general alteration in transmission in these animals, as input-output curves of stimulus versus fEPSP slope revealed no difference in basal synaptic transmission in knockouts compared to wild types (Figure 2E). Thus, these genetic data show that the effects of Iso on 5 Hz LTP primarily involve the $\beta 1$ subtype of AR.

β -AR Activation Facilitates LTP Elicited by Theta Frequency Stimulation in a PKA-Dependent, Macromolecular Synthesis-Independent Manner

The primary effector system activated by β -ARs is the PKA signaling system (reviewed by Rohrer and Kobilka, 1998). LTP elicited by pairing prolonged 5 Hz stimulation with β -AR activation is reduced by the PKA inhibitor

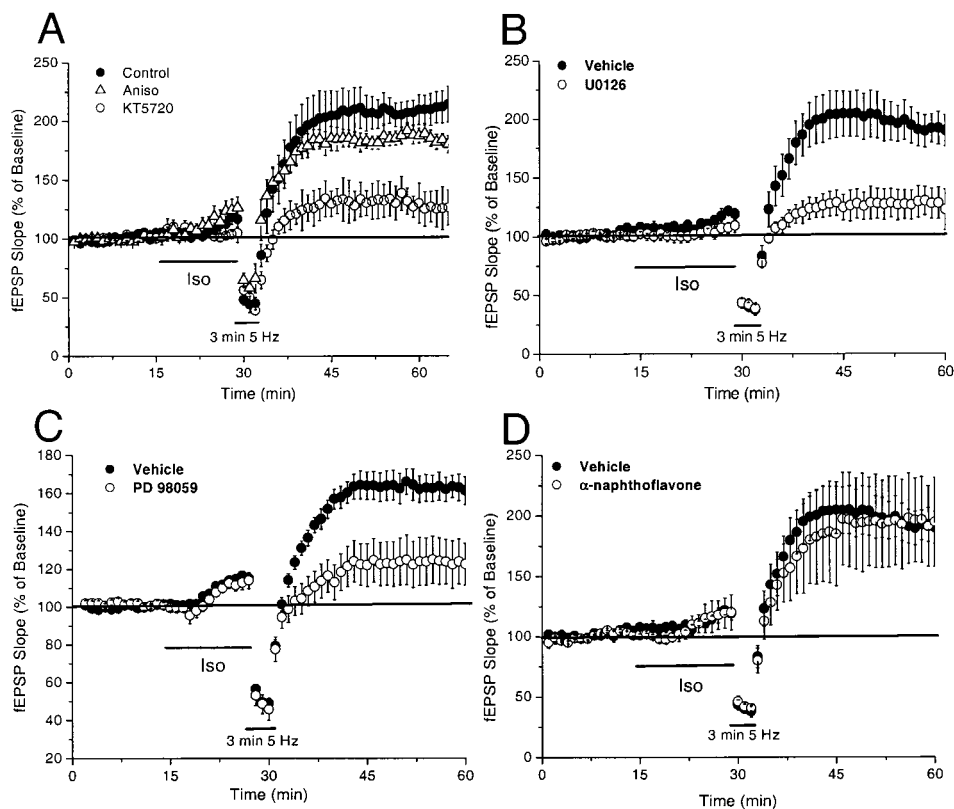


Figure 3. Inhibition of ERK Selectively Impairs the Ability of β -AR Activation to Facilitate the Formation of LTP by Prolonged Low-Frequency Stimulation

(A) LTP evoked by pairing Iso and 3 min of 5 Hz stimulation is PKA dependent but largely protein synthesis independent within the first hour after tetanus. LTP was evoked by pairing β -AR activation and 3 min of 5 Hz stimulation in the presence or absence of either 1 μ M KT5720 or 30 μ M anisomycin. Anisomycin was applied beginning 30 min before the administration of Iso and was removed 30 min after the end of the 3 min 5 Hz stimulus ($n = 6$, $p < 0.05$). KT5720 was applied for at least 1 hr before administration of Iso ($n = 3$).

(B and C) Effect of preincubation of hippocampal slices for 1 hr with (B) 20 μ M U0126, (C) 50 μ M PD98059 (vehicle, $n = 10$; PD98059, $n = 9$), or (D) 10 μ M α -naphthoflavone on the ability of 1 μ M Iso (application indicated by bar in each panel) and 3 min of 5 Hz stimulation to produce LTP.

KT5720 (1 μ M; Figure 3A). Similar results were reported previously with the PKA inhibitor H89 (Thomas et al., 1996). As was the case with LTP induced by brief 5 Hz stimulation, LTP was only very slightly reduced, within the first hour after tetanization, by preincubation of hippocampal slices with saturating concentrations of the protein synthesis inhibitor anisomycin for 30 min prior to administration of Iso (Figure 3A).

LTP Induced by Prolonged 5 Hz Stimulation Paired with β 1-Adrenergic Receptor Activation Is Specifically Reduced by MEK Inhibitors

Recent studies have shown by Western blot analysis that β -ARs in area CA1 can activate ERK (Roberson et al., 1999). To examine whether ERK also contributes to LTP elicited by prolonged 5 Hz stimulation when that stimulation is paired with activation of the β -AR, we pretreated slices with either the MEK inhibitor U0126 (20 μ M), PD 98059 (50 μ M), or 0.1%–0.3% DMSO. At this concentration, PD 98059 impairs both long-term facilitation of synaptic transmission at the sensory neuron–motor neuron synapse in *Aplysia* and LTP and phosphorylation of ERK in area CA1 of hippocampus (English and Sweatt, 1996, 1997; Martin et al., 1997). We found

that both PD98059 and U0126 reduced LTP without significantly affecting the acute enhancement of synaptic transmission by Iso prior to 5 Hz stimulation (Figures 3B and 3C). Further, pretreatment of hippocampal slices with both U0126 (20 μ M) and KT5720 (1 μ M) resulted in a similar level of inhibition ($133\% \pm 37\%$ of baseline, 35 min after 5 Hz stimulation, $n = 3$).

In addition to inhibition of MEK, PD 98059 has recently been reported to bind aryl hydrocarbon receptors (Reiners et al., 1998), which are present in CA1 pyramidal cells (Kainu et al., 1995). Thus, we tested the effects of a structural analog of PD 98059, α -naphthoflavone, on LTP elicited by pairing β 1-AR activation with prolonged 5 Hz stimulation. α -Naphthoflavone is a high-affinity aryl hydrocarbon receptor antagonist that, at the concentration used in this study (10 μ M), has no effect on the activation of ERK by β -ARs (Figures 6E and 6F). As shown in Figure 3D, at concentrations that are saturating for aryl hydrocarbon receptor antagonism, α -naphthoflavone administered at least 1 hr prior to Iso administration had no effect on the LTP elicited by this protocol.

CaMKII α can regulate synaptic plasticity in area CA1 of hippocampus by inducing a general shift in the frequency–response curve at this synapse (Mayford et

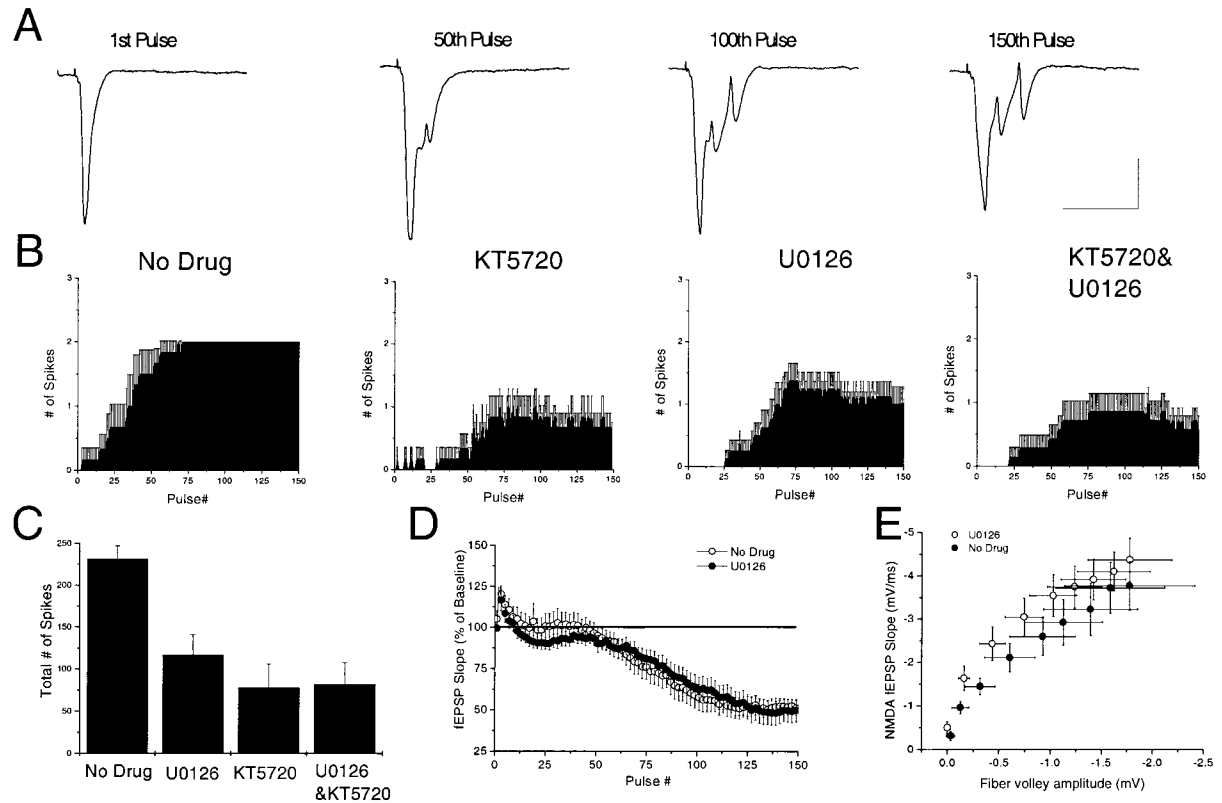


Figure 4. Regulation by ERK of CA1 Pyramidal Cell Spiking during Brief 5 Hz Stimulation

Scale is 2 mV and 10 ms.

(A) Representative fEPSPs at the indicated times during 5 Hz stimulation.

(B) Plots of the mean number of downward spikes in the fEPSP waveform during a 30 s 5 Hz tetanus (150 pulses) with no drug ($n = 6$), with KT5720 ($n = 8$), with U0126 ($n = 8$) (20 μ M), or with both KT5720 and U0126 ($n = 6$).

(C) Total number of spikes during a 30 s 5 Hz tetanus in the same conditions as (B).

(D) Plot of fEPSP slope during a 30 s 5 Hz tetanus.

(E) Input-output plots of stimulus intensity versus NMDA receptor-mediated fEPSP slope in hippocampal slices pretreated with vehicle or U0126 for 1 hr.

al., 1995). If ERK regulates LTP induced by 5 Hz stimulation and Iso by inducing a similar shift in the frequency-response curve, one might predict that inhibitors of ERK would also shift the response to prolonged 5 Hz stimulation alone in addition to reducing LTP induced by this stimulus. However, preincubation of hippocampal slices with U0126 had no effect on the response to a 3 min 5 Hz stimulation protocol in the absence of Iso (fEPSP percent of baseline 30 min after 3 min of 5 Hz stimulation: no drug, $113\% \pm 10\%$, $n = 5$; U0126, $121\% \pm 25\%$, $n = 5$).

ERK Regulates the Number of Postsynaptic Spikes Fired during Brief and Prolonged 5 Hz Stimulation

There are at least three ways by which ERK could regulate early components of LTP. First, ERK could regulate synaptic efficacy during the tetanus, producing a more robust depolarization to initiate LTP effector mechanisms more effectively. Indeed, activation of β -ARs can regulate glutamate receptors (Raman et al., 1996) and transiently enhance synaptic transmission (Gereau and Conn, 1994a). Second, ERK could regulate synaptic plasticity by modifying cellular excitability during the tetanus. For example, Iso reduces an afterhyperpolarization (AHP) and produces a slight depolarization of

CA1 pyramidal cells (Madison and Nicoll, 1986). Regulation of the AHP by β -AR activation has been proposed as a mechanism for regulation of LTP (Sah and Bekkers, 1996). Finally, ERK could participate directly in the downstream signal cascade necessary for the maintenance of LTP effector mechanisms.

To address the first possibility, we analyzed fEPSP slopes elicited during brief 5 Hz stimulation. We found that during the tetanus there was a short plateau of the fEPSP slope, followed by decay down to a steady-state level within the first 30 s of the tetanus, remaining at this level when 5 Hz stimulation was prolonged to 3 min (Figures 4D and 5D). In both cases, this fEPSP slope time course during 5 Hz stimulation was not significantly altered by MEK inhibitors. In addition, inhibitors of the ERK signaling cascade selectively decreased LTP elicited by the pairing of β -AR activation and 5 Hz stimulation without affecting the transient increase in transmission elicited by Iso prior to the tetanus (Figures 3B and 3C). Further, NMDA receptor-mediated fEPSPs (measured in the presence of 10 μ M DNQX and 450 μ M magnesium) were not reduced by pretreatment of slices with the MEK inhibitor U0126 (Figure 4E). Thus, β -AR activation and ERK are not enhancing the induction of

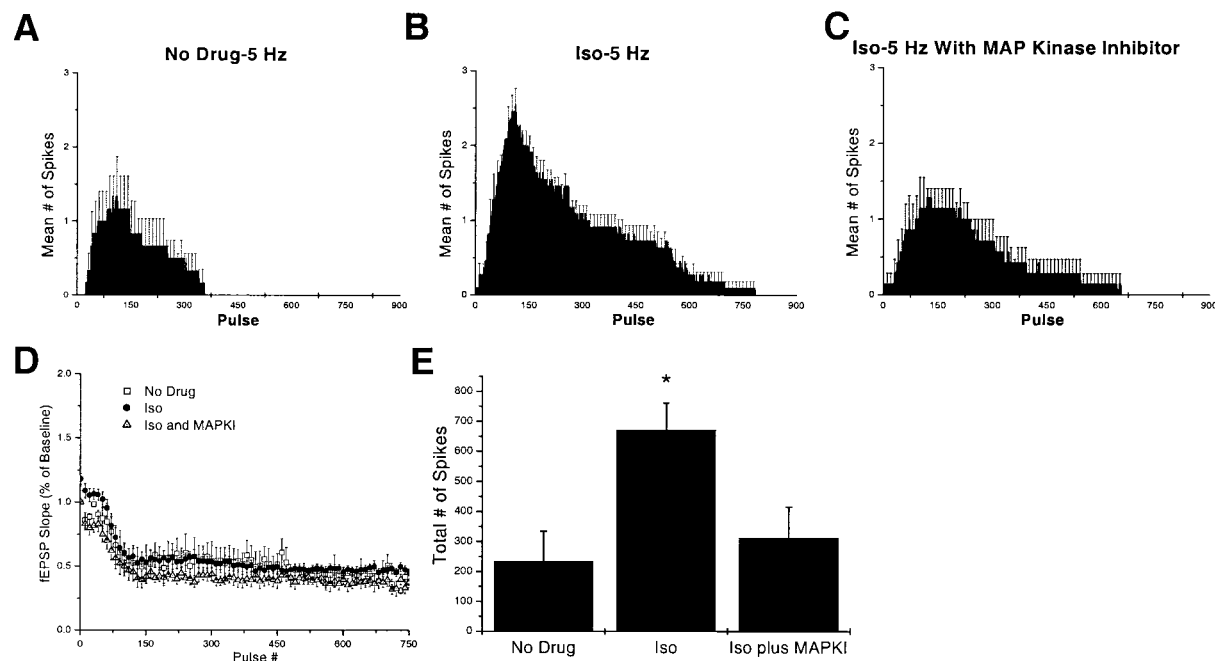


Figure 5. Regulation by Iso and ERK of CA1 Pyramidal Cell Spiking during Brief Low-Frequency Stimulation (A, B, and C) Plot of the mean number of downward spikes in the fEPSP waveform during a 3 min 5 Hz tetanus (900 pulses) with (A) no drug, (B) Iso, or (C) Iso and either PD 98059 or U0126. (D) Total number of spikes during a 3 min 5 Hz tetanus in the same conditions as (A), (B), and (C). (E) Plot of fEPSP slope during a 3 min 5 Hz tetanus with the same parameters as (A), (B), and (C).

LTP by altering glutamatergic transmission during the tetanus.

Intracellular recordings from CA1 pyramidal cells in hippocampal slices have demonstrated that during the beginning of the 5 Hz tetanus, postsynaptic neurons generate complex action potential firing patterns (Thomas et al., 1998) that resemble complex spikes recorded in vivo (Kandel and Spencer, 1961). These complex spikes dissipate during prolonged stimulation (see Figures 4A and 5A) and are time locked with clearly discernable spikes on the fEPSP waveform (Thomas et al., 1998). The number of complex spikes generated over time in response to 5 Hz stimulation is critical in determining the degree of LTP achieved (Thomas et al., 1998). Thomas et al. (1998) therefore suggested that these spikes might provide an amplification of the depolarization produced by 5 Hz stimulation, and thereby achieve activation of NMDA receptors to generate LTP.

To address the possibility that ERK regulates LTP by altering cellular excitability during the tetanus, we analyzed the number of spikes on the fEPSP during 5 Hz stimulation. Brief 5 Hz stimulation elicited an average of 231 ± 16 spikes, but only 117 ± 24 in the presence of U0126 ($20 \mu\text{M}$, $p < 0.002$; Figure 4B). The total number of spikes detected on fEPSPs during a 3 min 5 Hz tetanus increased ~ 3 -fold from 234 ± 100 to 670 ± 91 when $1 \mu\text{M}$ Iso was administered prior to 5 Hz stimulation ($n = 11$, $p < 0.01$ for no drug versus Iso; Figures 5A, 5B, and 5E). However, preincubation of slices with either PD 98059 or U0126 reduced the number of spikes elicited by combining $\beta 1$ -AR and 5 Hz stimulation to 311 ± 104 ($n = 8$, $p < 0.05$ Iso versus Iso plus MEK inhibitors;

Figures 5C and 5E). Thus, these data demonstrate that ERK regulation of LTP induced by 5 Hz stimulation involves the regulation of postsynaptic excitability.

LTP Induced by the Pairing of β -AR Activation and Prolonged 5 Hz Stimulation Is Synapse Specific

The finding that β -AR activation regulates postsynaptic excitability during theta frequency stimulation is consistent with previous studies showing that β -AR activation can block postsynaptic potassium conductances and depolarize CA1 pyramidal cells. In fact, activation of β -ARs can elicit a long-lasting increase in cellular excitability in the rat (Heginbotham and Dunwiddie, 1991; Gereau and Conn, 1994b). This long-lasting increase in postsynaptic excitability does not depend on NMDA receptor activation (Heginbotham and Dunwiddie, 1991). While regulation of postsynaptic excitability appears to play an important role in the synaptic enhancement elicited by pairing Iso and β -AR activation, this enhancement is mediated by NMDA receptor activation. As has been previously reported (Thomas et al., 1996), this enhancement was markedly reduced by the NMDA receptor antagonist D,L-AP5 ($105\% \pm 21\%$ of baseline 45 min after Iso administration in the presence of $100 \mu\text{M}$ D,L-AP5, $n = 5$). Thus, these data are consistent with the idea that regulation of postsynaptic excitability by $\beta 1$ -ARs converts the normally subthreshold 3 min 5 Hz tetanus into one that will elicit LTP by allowing for more effective activation of the NMDA receptor.

One way that postsynaptic excitability could be regulated is through an alteration in inhibitory transmission.

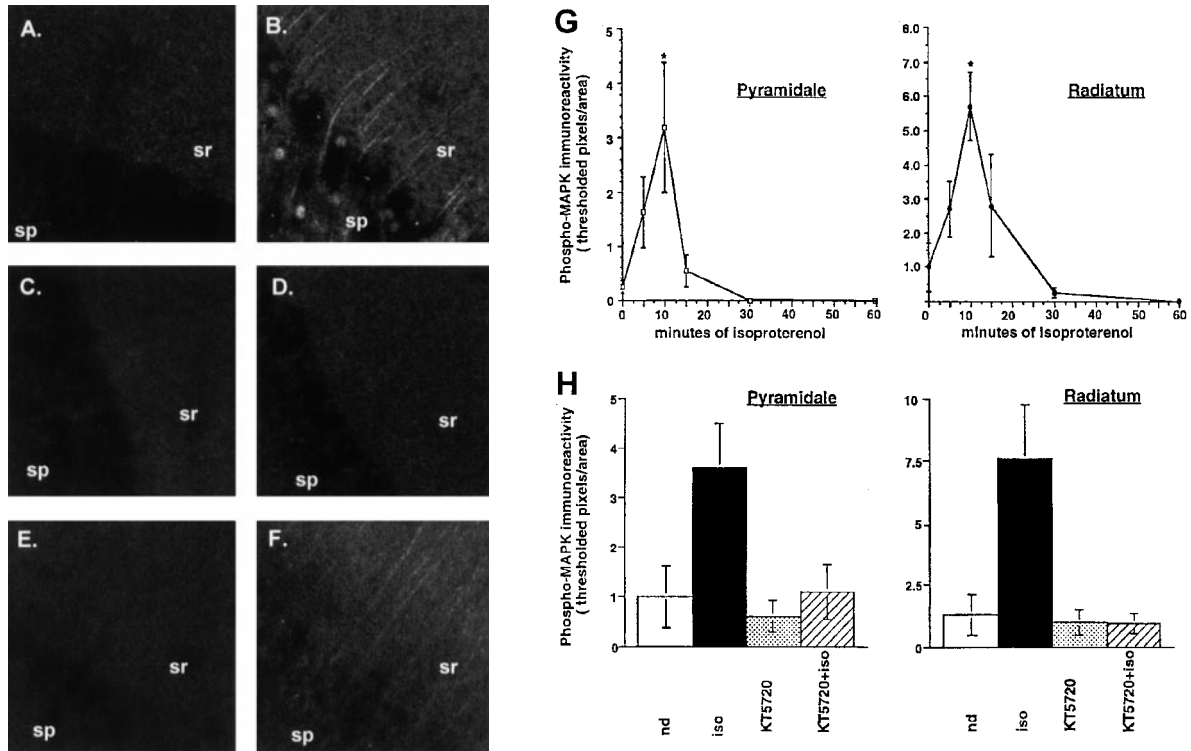


Figure 6. Iso Increases the Phosphorylation of ERK in Area CA1 of Hippocampus

(A–F) Representative images from area CA1 from experiments where slices were incubated with (A) no drug, (B) 1 μ M Iso, (C) 1 μ M KT5720, (D) 1 μ M Iso plus 1 μ M KT 5720, (E) 10 μ M α -naphthoflavone, or (F) 10 μ M α -naphthoflavone plus 1 μ M Iso and processed for anti-phosphorylated ERK immunoreactivity. Slices were pretreated for at least 1 hr with either vehicle, KT5720 or α -naphthoflavone, after which they were incubated in Iso for 10 min. Abbreviations: Sp, stratum pyramidale; sr, stratum radiatum.

(G) Time course of the effect of 1 μ M Iso on phosphorylation of ERK in area CA1 stratum radiatum and stratum pyramidale (average of five separate experiments, $n = 10$ slices for 0 min, 5 min, and 10 min; $n = 8$ slices for 15 min; $n = 6$ slices for 30 min and 1 hr).

(H) Quantification of the effects of Iso and KT5720 on ERK phosphorylation in area CA1. These data represent the mean values for nine separate experiments. It should be noted that in three of these nine experiments, no effect of Iso was observed on the phosphorylation of ERK.

For example, GABA_A receptor-mediated inhibitory postsynaptic potentials play a role in regulating dendritic action potential amplitude both in vitro and in vivo (Buzsaki et al., 1996; Tsubokawa and Ross, 1996). However, the lasting enhancement of CA1 pyramidal cell excitability induced by β -AR activation is not associated with obvious alterations in inhibitory transmission (Gereau and Conn, 1994b). We further found that coadministration of Iso and a 3 min 5 Hz stimulus evokes robust LTP in the presence of the GABA_A receptor antagonist picrotoxin (100 μ M, 193% \pm 47% of baseline, 50 min after initiation of Iso administration, $n = 4$). Thus, this LTP is likely to reflect a specific enhancement of excitatory transmission rather than downregulation of inhibitory transmission.

If ERK can regulate cellular excitability, is synapse specificity of evoked LTP maintained? This is particularly important since administration of Iso is likely to globally activate β -ARs on CA1 pyramidal cells. To examine the synapse specificity of this LTP, we used two stimulating electrodes positioned equidistantly around a common recording electrode to activate two independent populations of CA1 pyramidal cell synapses in the slice. We found that while administration of Iso alone elicited a slight enhancement of synaptic transmission at both synapses, NMDA receptor-dependent LTP was

only evoked in the pathway that underwent stimulation for 3 min at 5 Hz (126% \pm 20% of baseline 50 min after initiation of Iso administration in nontetanized pathway, 176% \pm 30% of baseline 50 min after initiation of Iso administration in tetanized pathway, $n = 4$).

β -AR Activation Stimulates the Rapid Phosphorylation of ERK in the Dendrites of CA1 Pyramidal Cells in a PKA-Dependent Fashion

The finding that ERK participates in regulating postsynaptic responses during the tetanus suggests that this ERK must be activated by β receptor activation even before 5 Hz stimulation begins. Thus, we wondered whether Iso activated ERK with a time course and in a location consistent with an ability to play a role in this regulation. To determine the spatial and temporal patterns of ERK activation elicited by β -ARs in area CA1, we used confocal imaging to examine the labeling of hippocampal slices with an anti-phosphorylated ERK antibody. Hippocampal slices had a relatively low (but not undetectable) level of phosphorylated ERK 3–4 hr after dissection. Iso (1 μ M) induced a rapid increase in phospho-ERK immunoreactivity in both the dendritic shafts and in the cell bodies of pyramidal cells in area CA1 (Figures 6A, 6B, 6G, and 6H). This activation of ERK by Iso appears to require PKA. Preincubation of

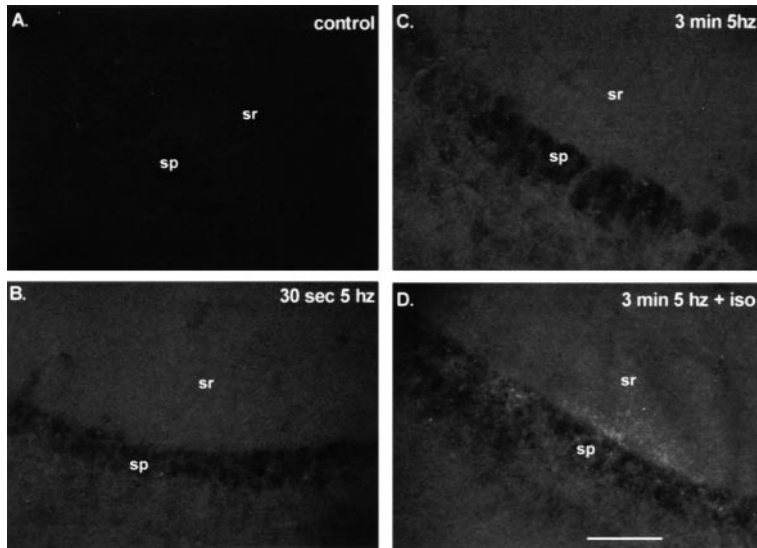
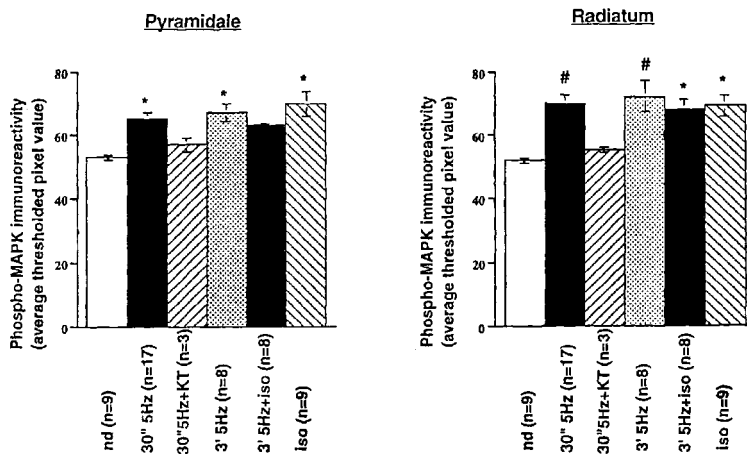


Figure 7. 5 Hz Stimulation Protocols Evoke Phosphorylation of ERK in Hippocampal Slices

(Top) Representative images from area CA1 from experiments where slices received (A) test stimulation, (B) 30 s of 5 Hz stimulation, (C) 3 min of 5 Hz stimulation, or (D) 3 min of 5 Hz stimulation after 15 min of Iso. Abbreviations: sr, stratum radiatum; sp, stratum pyramidale.

(Bottom) Quantification of these results (* $p < 0.05$, # $p < 0.01$).



hippocampal slices for 1 hr with the PKA inhibitor KT5720 (1 μ M) significantly reduced the levels of phosphorylated ERK in response to Iso (Figures 6C, 6D, and 6H). As mentioned above, we found that the aryl hydrocarbon receptor ligand α -naphthoflavone (10 μ M) did not impede the ability of Iso administered for 10 min to activate ERK (Figures 6E and 6F; α -naphthoflavone alone, 100% \pm 51% of α -naphthoflavone treated slices; α -naphthoflavone plus 1 μ M Iso for 10 min, 187% \pm 22%; $p < 0.001$).

Phosphorylation of ERK in area CA1 of hippocampus by β -AR activation is rapid and transient. We detected phosphorylated ERK within 5 min of Iso administration. This phosphorylation peaked at 10 min and returned to baseline by 1 hr after administration of Iso (Figure 6G). Thus, ERK is activated by β -ARs with a time course that is consistent with the regulation by ERK of postsynaptic excitability during 3 min 5 Hz stimulation.

We also examined whether 5 Hz stimulation—brief, prolonged, and prolonged in conjunction with Iso—affected ERK phosphorylation 30–60 s after the tetanus. Interestingly, we found that each of these conditions results in a similar level of phosphorylation of ERK compared to test stimulated slices (Figure 7). ERK phosphorylation induced by 30 s of 5 Hz stimulation was blocked

by pretreatment of slices with 1 μ M KT5720, suggesting the involvement of PKA in its activation (Figure 7). The finding that prolonged 5 Hz stimulation activates ERK but does not elicit LTP suggests that although ERK is necessary, it is, by itself, not sufficient to induce LTP. In fact, it seems unlikely that ERK activated by the tetanus could play a role in regulating neuronal excitability during that same tetanus.

Double Labeling Experiments Suggest that β -ARs Activate ERK in Proximal Dendrites and Cell Bodies of CA1 Pyramidal Cells

The labeling by anti-phosphorylated ERK antibodies suggests a dendritic and somatic localization of the activated ERK. To confirm this localization, we performed double labeling experiments using anti-MAP2 and anti-synaptophysin antibodies as postsynaptic and presynaptic markers, respectively. As shown in Figure 8, phospho-ERK immunoreactivity induced by β -AR activation colocalized with MAP2 immunoreactivity. In contrast, there was no significant colocalization between phospho-ERK and synaptophysin immunoreactivity (data not shown). Thus, these data suggest that ERK is activated rapidly in an appropriate location to participate in regulating the induction of early forms of LTP.

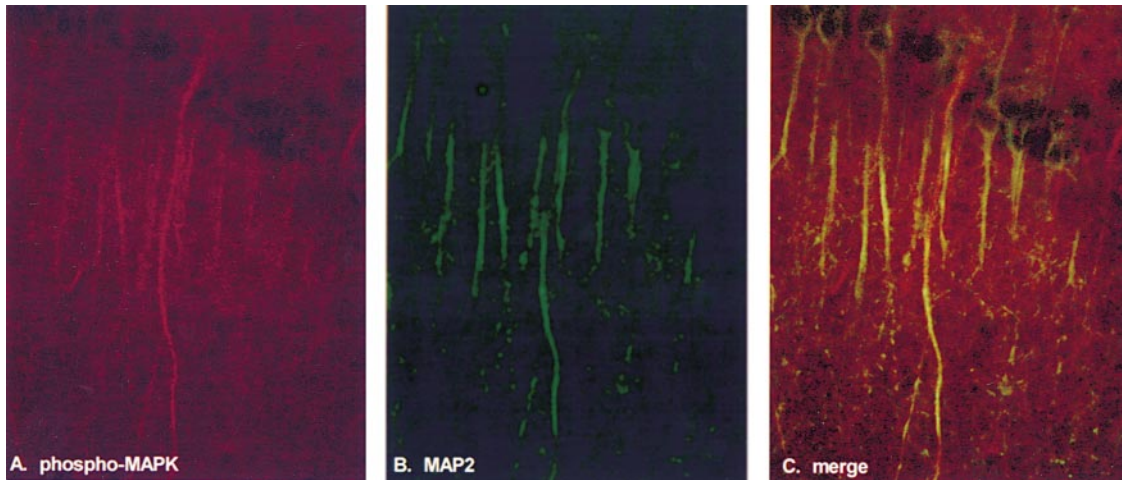


Figure 8. Double Labeling Analysis of the Subcellular Localization of ERK Phosphorylated by β -AR Activation (A and B) Representative experiments in which the same slice is probed for the localization of phosphorylated ERK and MAP2, respectively. (C) A pseudo-color merging of the two images. Yellow indicates regions of signal overlap.

Discussion

NMDA Receptor-Dependent LTP Induced by Physiologically Relevant Theta Stimuli Has Unique Properties

The theta rhythm (3–12 Hz) of the EEG recorded in the hippocampus is generated in part by septal afferents. This rhythm causes CA1 pyramidal cells to fire complex spikes, a pattern of firing that is recruited when an animal moves through space (Oddie and Bland, 1998). The firing of complex spikes by pyramidal cells is thought to be critical for an animal to solve tasks that require the integration of complex sensory information. Despite the potential behavioral importance of theta frequency-generated LTP, most studies of LTP have focused not on LTP induced by theta frequency stimuli but on LTP induced more artificially, either by high-frequency tetanization (100 Hz) or by prolonged somatic depolarization paired with low-frequency stimulation. One reason that these artificial patterns of stimulation have been used so extensively is that it has generally been assumed that since both are dependent on NMDA receptor activation, the molecular mechanisms of LTP produced by high-frequency stimuli are the same as those produced by more physiological theta frequency stimulation. Surprisingly, Thomas and colleagues (1996) found that LTP produced by brief theta frequency stimulation is completely dependent upon PKA. We have extended these findings by showing that, in addition to requiring PKA, the LTP elicited by theta frequency stimulation differs from 100 Hz LTP by also requiring activated ERK.

ERK Plays a Role in the Postsynaptic Response during Theta Frequency Stimulation by Regulating the Generation of Complex Spikes

Our data suggest that one mechanism by which ERK regulates LTP induced by theta frequency stimulation is through the regulation of spike burst generation by the postsynaptic cell during the tetanus. In vivo CA1

pyramidal cells fire in bursts of action potentials known as complex spikes (Kandel and Spencer, 1961). These spikes have been postulated to be an important means for regulating synaptic efficacy in vivo (reviewed by Lisman, 1997), but it has proven difficult to recapitulate these spikes in hippocampal slices. Recently, Thomas et al. (1998) found that complex spikes can be elicited in CA1 pyramidal cells in slices when theta frequency stimulation is employed and that these complex spikes appear to be critical for the generation of LTP by theta frequency stimulation (Thomas et al., 1998). Our results show, surprisingly, that these complex spikes can be regulated in vitro by ERK in a manner that correlates with the participation of ERK in LTP. Thus, these data suggest a new role for ERK in regulating LTP. Whereas several labs have now shown that ERK is important for LTP elicited by repeated high-frequency tetanization (English and Sweatt, 1997; Atkins et al., 1998; Impey et al., 1998; Coogan et al., 1999), English and Sweatt found no effect of ERK inhibitors on the synaptic responses elicited during the high-frequency tetanus. Thus, in addition to its roles in initiating gene transcription, our studies show that ERK can also play a role in the regulation of more transient forms of neuronal function.

Recording from two independent pathways in area CA1, we found that the independent, nontetanized pathway was not potentiated in response to 5 Hz tetanization in the presence or absence of Iso. Thus, even though the activation of ERK by Iso is cell-wide, some degree of synapse specificity is maintained. Perhaps backpropagating action potentials initiated in the cell soma invade dendrites and play a role in amplification of depolarization by weak stimuli to allow effective depolarization and thus activation of NMDA receptors of tetanized synapses (reviewed by Magee et al., 1998). In fact, the downward deflection of "complex spikes" on the fEPSPs recorded in the stratum radiatum is consistent with a dendritic "origin." Dendritic action potentials are subject to modulation, and β -AR activation doubles the amplitude of these spikes in a PKA-dependent manner (Hoffman and Johnston, 1999).

Activation of β -ARs Increases ERK Phosphorylation in CA1 Pyramidal Cells

We find that activation of β -ARs results in a rapid and transient activation of ERK in the dendrites and cell bodies of the CA1 pyramidal cells. Thus, the timing and location of ERK activation are consistent with its involvement in the regulation of complex spiking and LTP elicited by application of Iso followed by 5 Hz stimulation.

Surprisingly, we also found that both brief and prolonged 5 Hz stimulation increase ERK phosphorylation, even in the absence of Iso. While this ERK is not likely to participate in the regulation of neuronal excitability during the tetanus, it is particularly intriguing that 3 min of 5 Hz stimulation in the absence of Iso, a protocol that does not elicit LTP, nonetheless resulted in a level of ERK phosphorylation that was indistinguishable from the level evoked by stimuli that do elicit LTP. These data suggest that ERK activation is not sufficient for the generation of LTP. Previous studies by Thomas et al. (1996) suggested that one reason that 3 min of 5 Hz stimulation does not evoke LTP is that the longer stimulus procedure evokes more significant activation of phosphatases, which counteract phosphorylation by kinases necessary for LTP. For example, Thomas et al. (1996) and we (D. G. W. and E. R. K., unpublished data) have found that pretreatment of hippocampal slices with the phosphatase inhibitor calyculin A can substitute for application of Iso. If this is the case, then even though ERK is activated by this stimulus, it may be less effective because some substrates of ERK may be affected by the increased phosphatase activity. Thus, in addition to activating ERK directly, β -ARs may play a second important role in regulating ERK signaling indirectly by suppressing phosphatase activity.

In Neurons, β -ARs Can Recruit Kinases Other Than PKA to Regulate Cellular Excitability and LTP

In addition to showing that β -ARs can activate ERK, our data show that activated ERK plays a role in the facilitation of LTP by β -AR activation. To determine the specific subtype of β -AR involved in this effect of Iso, we examined the ability of Iso to elicit LTP when paired with a 3 min 5 Hz tetanus in β 1-, β 2-, and β 1/ β 2-AR knockout mice. We found that the responses to Iso were completely abolished in β 1- and β 1/ β 2-AR knockout mice but not in β 2-AR knockouts. Thus, the β 1-AR is required for Iso-induced facilitation of LTP by prolonged 5 Hz stimulation in area CA1 of hippocampus. These data are consistent with previous pharmacological studies that suggested that some of the effects of Iso on postsynaptic excitability in area CA1 are likely to be mediated through a β 1-like receptor (Fowler and O'Donnell, 1988). Since other subtypes of β -ARs are likely to be present in CA1 pyramidal cells, multiple β -AR subtypes may couple to ERK in these cells.

The cAMP signaling cascade can facilitate the activation of ERK by several means, including PKA-mediated phosphorylation of an adaptor molecule, rap1, which then activates ERK through a B-raf-dependent cascade (Vossler et al., 1997); by the direct binding of cAMP to guanine nucleotide exchange factors for rap1 (de Rooij et al., 1998; Kawasaki et al., 1998); and through a novel

mechanism in which PKA, activated by the β -AR, phosphorylates the receptor and switches the coupling specificity of its G protein such that the receptor can now bind and activate G_i (Daaka et al., 1997).

Our results with KT5720 suggest that PKA is required for the activation of ERK by β -ARs. Although the specificity of this kinase inhibitor has been challenged (Olsen et al., 1998; but see, for example, Iwasaki et al., 1998), similar results have been reported with H89, another PKA inhibitor (Thomas et al., 1996; Roberson et al., 1999). In addition to the mechanisms listed above by which PKA can activate ERK, we and others have found that one role of PKA in LTP at CA1 synapses is to suppress an endogenous phosphatase cascade (Blitzer et al., 1995, 1998; Thomas et al., 1996; Mansuy et al., 1998; Winder et al., 1998). Inhibition of phosphatases with okadaic acid results in the activation of ERK in hippocampal neurons (Runden et al., 1998). While these data and our electrophysiological results with coapplication of both MEK and PKA inhibitors suggest that there is considerable overlap in the function of PKA and MEK in regulating LTP, these data do not rule out the possibility that these kinases also have nonoverlapping roles.

The noradrenergic modulatory system that originates in the locus ceruleus is thought to play an important role in attention (Coull, 1998). Since prolonged 5 Hz stimulation only elicits LTP in the presence of β -AR activation, it is tempting to suggest that this represents an adaptation to facilitate LTP during attentive states. To determine the behavioral relevance of the function of the β 1 receptor in area CA1, it will be important to use the Cre-lox system to generate mice lacking the β 1-AR only in CA1 pyramidal cells (Tsien et al., 1996).

Behaviorists have known for almost a century that the outcome of a learning experiment is critically dependent on the specific protocol used. Slight changes in protocol can produce either different behavioral outcomes or phenotypically similar outcomes that are mediated by mechanistically different combinations of processes. A body of evidence now suggests that synapses that undergo LTP, such as the Schaffer collateral synapse, in fact can undergo a family of phenotypically similar but mechanistically quite different synaptic changes. Thus, even NMDA receptor-dependent LTP does not necessarily imply a single mechanism, making it critical that several different protocols for eliciting LTP be employed when examining the effects of a drug or genetic manipulation.

Experimental Procedures

Electrophysiology

Transverse hippocampal slices were prepared as previously described (Winder et al., 1998). C57b6 mice (Jackson) of either sex, aged 7–18 weeks, were used. Hippocampi were sliced (400 μ m), placed in oxygenated ACSF (NaCl, 124 mM; KCl, 4.4 mM; CaCl₂, 2.5 mM; MgSO₄, 1.3 mM; NaH₂PO₄, 1 mM; glucose, 10 mM; and NaHCO₃, 26 mM), subfused (1–2 ml/min) in an interface chamber and allowed to equilibrate for 60–90 min at 28°C.

For extracellular recordings, ACSF-filled glass electrodes (1–3 M Ω) were positioned in the stratum radiatum of area CA1. A bipolar nichrome stimulating electrode was also placed in stratum radiatum for stimulation of Schaffer collateral afferents (0.05 ms duration). Unless otherwise mentioned, test stimuli were applied at a frequency of 1 per minute (0.017 Hz) and at a stimulus intensity that elicits an

fEPSP slope that was 45% of the maximum. Experiments in which changes in the fiber volley occurred were discarded. Drugs were applied through the perfusion medium. In experiments in which downwardly deflecting spikes on fEPSPs were quantified, it is important to note that this quantitation excluded the first peak of the fEPSP because of the difficulty in resolving a downward spike from the rising phase of the EPSP. Thus, our counts of these spikes are likely to be significant underestimates. KT5720 was purchased from Research Biochemicals International. Iso and α -naphthoflavone were purchased from Sigma Chemicals. PD 98059 was purchased from Calbiochem, and U0126 was kindly provided by Dr. James Trzaskos (Merck). PD 98059 and U0126 were dissolved in DMSO and then diluted in ACSF (0.1% vehicle).

Immunocytochemistry

Tissue preparation for the analysis of the location of phosphorylated ERK was identical to that for slices prepared for electrophysiological recordings. To examine the time course of Iso phosphorylation of ERK after dissection, slices were maintained in a submerged, oxygenated holding chamber at room temperature for at least 3 hr before Iso was applied. After drugs were administered for appropriate lengths of time, slices were removed from the holding chamber and placed in 4% paraformaldehyde containing 2 mM each of sodium orthovanadate and sodium fluoride. For experiments examining the effects of 5 Hz stimulation on ERK phosphorylation, after dissection slices were placed in an interface chamber at 28°C and allowed to equilibrate for 3 hr. Following equilibration, recording and stimulating electrodes were placed in area CA1 as in the electrophysiological experiments, a stable baseline of 40%–50% of the maximal fEPSP slope was obtained, and then the slices were either removed for fixation as above, stimulated for 30 s at 5 Hz, or stimulated for 3 min at 5 Hz. In some cases, slices were pretreated with KT5720 or vehicle for 1 hr before stimulation, or they were treated with Iso for 15 min, following which the slices were either removed for fixation or stimulated for 3 min at 5 Hz prior to fixation.

Phosphatase inhibitors were then carried through all subsequent slice manipulations. After a 1 hr fixation at 4°C, the slices were rinsed in phosphate buffer (PBS) and permeabilized with 0.1% Triton X-100 for 1 hr at room temperature in PBS. After washing in PBS, slices were placed in PBS containing 50 mM NH₄Cl for 15 min at room temperature to quench free aldehydes. After additional washes in PBS, nonspecific staining was reduced by incubating slices with 10% goat serum in PBS for 1 hr at room temperature. Slices were incubated with primary antibody (anti-phospho-MAP kinase, ERK1 and 2, New England Biolabs) for 24 hr at 4°C and rinsed overnight in PBS and incubated with secondary antibody (Cy3 goat anti-rabbit, Jackson Immunoresearch) diluted in 10% goat serum for 24 hr at 4°C. Slices were then washed extensively with PBS. In double label experiments, mouse monoclonal antibodies (anti-MAP2 [Sigma] or anti-synaptophysin [Roche-Boehringer Mannheim]) were included in the primary antibody incubation, and Cy2 goat anti-mouse antibodies were included in the secondary incubation.

Confocal Imaging and Quantitation of Label Intensity

Slices were imaged on a Biorad MRC 1000 scanning laser confocal microscope equipped with an argon krypton laser. Images were taken using a 40 \times water immersion objective (NA 0.75) with 30% of laser light, iris of 8, and gain of 1400. Z series consisting of stacks of eight images at 2.5 μ m steps were taken of the CA1 pyramidal cell region of each hippocampal slice. To image slices that had been electrically stimulated, we selected the brightest region in CA1 and then took stacks of eight images taken at 2.5 μ m steps of this region. Quantification was done on projections of these Z series using Universal Imaging Metamorph software. To quantitate changes in dendritic staining, images were thresholded to a pixel value of 50, so that only positively stained dendritic structures were quantified within a field. The average pixel intensity above 50 was then divided by the total area to determine integrated pixel density in dendritic structures. Integrated pixel density was thus determined in the stratum radiatum and in the stratum pyramidale of each slice. The statistical significance of changes in immunoreactivity with different treatments was determined by ANOVA followed by Newman Kuel multiple range test.

Acknowledgments

We thank Dr. James Trzaskos at Dupont for supplying us with U0126, as well as Susan Patterson, Paul Pavlides and Steve Siegelbaum for helpful comments on an earlier version of the manuscript.

Received March 12, 1999; revised September 23, 1999.

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