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Phosphorylation and Local Presynaptic Protein Synthesis in Calcium- and Calcineurin-Dependent Induction of Crayfish Long-Term Facilitation

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Summary

Long-term facilitation at the crayfish opener muscle is elicited by prolonged high frequency stimulation, and arises from an increase in functional active zones, resulting in increased transmitter release. LTF induction depends critically upon presynaptic calcium accumulation and calcineurin (PP2B) activity. The protein synthesis dependence of this synaptic strengthening was investigated. LTF occurred without transcription, but the translation inhibitors cycloheximide and anisomycin, or local presynaptic injection of mRNA cap analog m^7GpppG, impaired LTF expression. Both MAP kinase and phosphatidylinositol 3-OH kinase (PI3K) activation are implicated in this rapamycin-sensitive synaptic potentiation. This study defines an important role for protein synthesis in the expression of activity-dependent plasticity, and provides mechanistic insight for the induction of this process at presynaptic sites.

Introduction

Crustacean neuromuscular synapses exhibit both short-term and long-term alterations in functional state. One such long-term alteration is long-term facilitation (LTF), a synaptic enhancement following prolonged high frequency stimulation lasting several hours (Atwood et al., 1975), or 1–2 days in vitro (Lnenicka and Atwood, 1985). Numerous data support a presynaptic locus of action, with "silent" synapses on the motor nerve terminal shifting to an "active" state following tetanic stimulation (Wojtowicz and Atwood, 1985, 1986, 1988; Wojtowicz et al., 1988). Ultrastructurally, LTF is not associated with an increase in either total synaptic vesicle counts or apparently docked vesicles compared to unstimulated controls, but a modest increase in the number of active zones per synapse (defined as dense bodies with clustered synaptic vesicles) has been observed, and an increase in the number of release sites was inferred (Wojtowicz et al., 1994).

In the present study, we find that presynaptic calcium entry during a tetanus, and calcineurin activity throughout conditioning stimulation, is essential for the induction of LTF, but not its maintenance. Conversely, the classical kinases thought to be involved in mammalian long-term plasticity, such as the adenylyl cyclase/ PKA pathway, PKC, and calcium/calmodulin-dependent protein kinase II (CaMKII) do not appear to play any role in this form of synaptic plasticity.

The protein synthesis dependence of LTF was investigated. The requirement for de novo protein synthesis in long-term synaptic plasticity has been repeatedly demonstrated in both vertebrate and invertebrate neuronal preparations, but both the regulation, cellular location, and final protein products of this process remain imperfectly characterized (Schuman, 1999). The regulation of protein synthesis may result from the induction of nuclear transcription and mRNA synthesis, or at the level of translation of preexisting mRNA to protein, or both. Proteins may be synthesized in the somata of cells, and transported to their site of action (the synapse), or may be synthesized in situ. Finally, protein synthesis may occur at either or both presynaptic and postsynaptic sites. Our results indicate that LTF expression requires local presynaptic protein translation, implying that protein is synthesized close to release sites from preexisting mRNA.

The regulation of translation initiation has been an area of intense study, primarily in non-neuronal cells (reviewed by Dufner and Thomas, 1999; Gingras et al., 1999b; and Gray and Wickens, 1998). Translation initiation is a complex, multistep process involving a large number of protein factors and multiprotein complexes, in addition to ribosomes. Numerous components of the translational machinery are phosphoproteins, and an emerging picture of translational regulation has underscored the importance of phosphorylation/dephosphorylation events in translation, especially of the eIF4 and eIF2 eukaryotic initiation factors and their regulatory proteins.

Several separate and converging kinase pathways have been identified which appear to regulate translational control, involving phosphatidylinositide-3OH kinase (PI3K) signaling through PKB/Akt, the mammalian target of rapamycin kinase FRAP/mTOR and the 40 S ribosomal protein S6 kinases, as well as the ras/raf/MAP kinase and atypical PKC pathways. Most notable in respect to the present study, our results indicate that LTF is exquisitely sensitive to both PI3K and MAP kinase inhibition. Additionally, LTF is rapamycin sensitive, indicating that translational control at presynaptic sites is likely mediated by the phosphorylation state of the same initiation factors that have been shown to be so crucial in translational control during cell growth and development. The finding in the present study that these pathways are activated following calcium accumulation and calcineurin activation is intriguing, and represents a novel mechanism for induction of protein synthesis during activity-dependent plasticity.

Results

Characterization of LTF

After establishing baseline amplitudes of EJPs from proximal muscle fibers in response to a 2 Hz axonal

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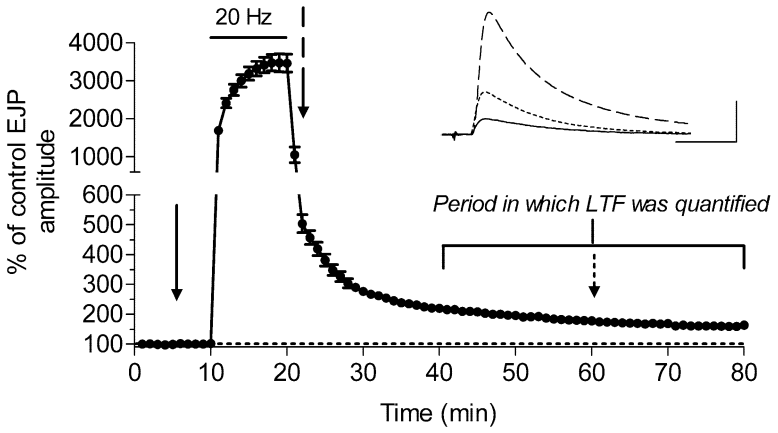

Figure 1. Quantification of LTF

EJP amplitude was normalized to average amplitude recorded between 0–10 min at 2 Hz stimulation frequency. At 20 Hz, EJP amplitude increased as a result of calcium-dependent facilitation, augmentation, and potentiation. At 2 Hz following high frequency stimulation, EJP amplitude decayed bi-exponentially to a plateau greater than the control pretetanic EJP amplitude, indicative of long-term facilitation. The **inset** illustrates EJPs from one preparation. The line-style of each trace corresponds to the time point indicated by the matching arrow. Scale bar 1 mV, 25 ms.

stimulus, long-term facilitation was elicited following a 20 Hz, 10 min tetanic train (Figure 1). During the tetanus, EJP amplitude was elevated to 3430 ± 220% of control amplitude, a process well described as due to the various calcium-dependent processes of facilitation, augmentation, and potentiation (Zucker, 1999). Immediately following the tetanus, EJP amplitude decayed bi-exponentially (τ_{decay1} = 11.5 ± 0.5 s, τ_{decay2} = 7.1 ± 0.9 min, n = 9), representing augmentation and post-tetanic potentiation (PTP), respectively. Facilitation decays too rapidly to be detected by our sampling rate. Rather than decay back to pre-tetanic control levels, EJP amplitude characteristically remained enhanced for the remainder of the recording, and this was attributed to long-term facilitation (LTF). To quantify LTF in the absence of PTP, we measured enhancement of transmission between 20 and 60 min post-tetanus, which was 82 ± 8% (n = 51, $p < 0.0001$, Student's paired t test).

Requirement for Local Protein Synthesis

Long-term changes in synaptic strength have been linked to the necessity for local protein synthesis in order to generate, or maintain, an enhanced level of synaptic activity. We investigated whether LTF generation required such a process. Figure 2A shows that the protein translation inhibitor anisomycin (20 μM, n = 4), applied 30 min prior to and throughout the experiment, almost abolished LTF, giving only an 8 ± 6% increase in EJP amplitude compared to a control increase of 60 ± 11% during LTF (n = 11, $p < 0.05$). Treatment with cycloheximide (60 μM–100 μM, n = 8) was also effective (Figure 2B), resulting in only a 26 ± 17% increase in EJP amplitude during the LTF expression period, compared to control increases of 87 ± 16% (n = 16, $p < 0.05$). Neither of these agents had any effect on the calcium-dependent forms of synaptic plasticity observed during the tetanus (Figures 2A and 2B). Furthermore, a mixture of cycloheximide (60 μM) and anisomycin (20 μM) applied 30 min prior to and throughout a 90 min recording did not affect basal transmission (n = 3, Figure 2C), demonstrating that the protein synthesis inhibitors were not interfering with processes required to maintain synaptic transmission throughout the time observed.

Based on analysis of binomial release parameters and ultrastructural data, the locus of LTF has been shown to be purely presynaptic (Wojtowicz and Atwood, 1985, 1986, 1988; Wojtowicz et al., 1988). The presynaptic cell bodies of crayfish walking leg motor neurons are located in abdominal ganglia, so the in vitro neuromuscular preparation of the isolated walking leg lacks presynaptic cell bodies. Therefore, induction of protein synthesis during LTF in vitro could not possibly occur at the level of presynaptic nuclear transcription. Nevertheless, we investigated the possible role of postsynaptic transcription in LTF by bath application of the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, 75 μM). As expected, a 30 min preincubation of DRB and its presence throughout the recording shown in Figure 2D did not affect EJP amplitude increase during LTF compared to time-matched controls, being 151 ± 63% in DRB (n = 5) compared to a control LTF increase of 142 ± 57% (n = 6, $p > 0.7$).

Protein Synthesis Occurs Close to Presynaptic Release Sites

The presynaptic versus postsynaptic locus of protein synthesis was confirmed by local pressure injection of the mRNA cap analog m^7GpppG into the presynaptic axon. The cap analog inhibits protein synthesis by competing with endogenous capped mRNA for the eukaryotic initiation factor eIF4E. Binding of eIF4E to mRNA facilitates translation initiation, (reviewed in Gingras et al., 1999b), and interference of this binding by addition of excess m^7GpppG has been shown to interfere with protein synthesis (Huber et al., 2000). Filling of presynaptic boutons with m^7GpppG was monitored by coinjection of fluorescein (Figure 3A). This filling procedure did not alter either the resting membrane potential of the axon, or action potential shape (data not shown). When the concentration of m^7GpppG in a visually identified bouton overlying a particular muscle cell was estimated to be between 130 and 200 μM, the enhancement of synaptic strength recorded at that muscle cell following an LTF-inducing tetanus was examined. Figure 3B shows the increase in synaptic strength recorded 20–60 min post-tetanus and compared to pretetanus levels was only 40 ± 14% in m^7GpppG injected axons (n = 7), compared to an increase of 132 ± 21% in time-matched controls (n = 8, $p < 0.005$). Axons injected with similar quantities of fluorescein-containing carrier solution alone exhibited LTF no different from controls (n = 2, data not shown). This data lends strong support to the notion

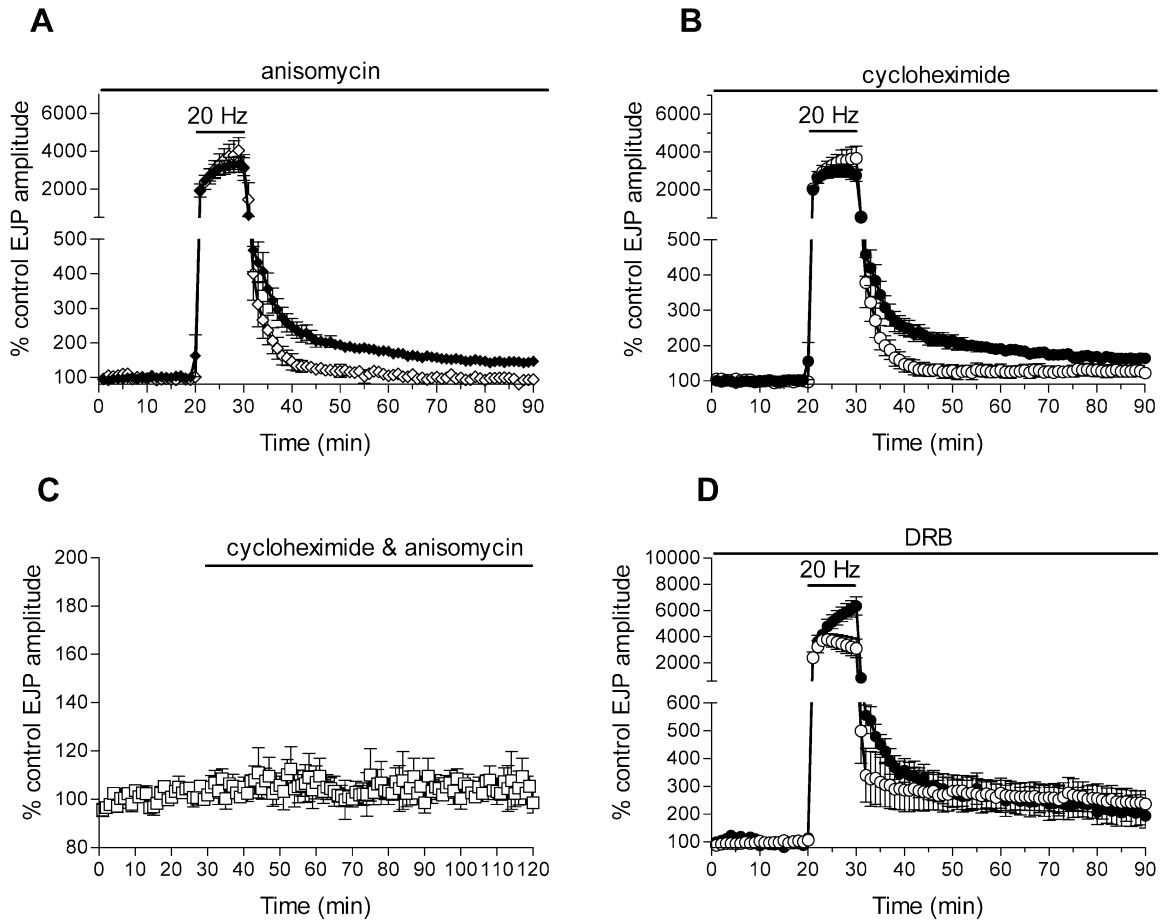

Figure 2. LTF Expression Requires Protein Translation but Not Transcription

(A) The protein synthesis inhibitor anisomycin (20 μM) bath applied 30 min prior to and throughout the time shown significantly reduced LTF (◇) compared to controls performed within the same batch of crayfish (◆). Tetanic enhancement of EJPs was unaffected.

(B) Bath application of cycloheximide (60–100 μM) for the same time period as anisomycin also significantly reduced LTF (o) compared to matched controls (•).

(C) Control experiment demonstrating that a mixture of anisomycin (20 μM) and cycloheximide (60 μM) applied 30 min prior to and throughout the time shown did not affect basal synaptic strength.

(D) Bath application of the transcription inhibitor (DRB, 75 μM) had no effect on LTF (o) compared to matched controls (•).

that the translational machinery is located within the area of m^7GpppG diffusion, i.e., within presynaptic terminals, or in axons within several hundred microns of release sites.

Conversely, pressure injection of a muscle fiber with m^7GpppG (~90 – 170 μM, n = 4) did not significantly affect the amplitude of LTF recorded from that fiber when compared to control fibers injected with the fluorescein-carrier solution alone (n = 3; Figures 3C and 3D).

Intracellular Calcium Accumulation May Be Required for LTF Induction

We next sought to investigate the mechanism of LTF induction, and hence a possible cue for initiation of protein translation. Intracellular calcium accumulation is responsible for all reported forms of activity-dependent short-term plasticity at the crayfish neuromuscular junction (Zucker, 1999), and thus we were interested in determining whether calcium also played a role in LTF. The intracellular calcium level, $[Ca^{2+}]_i$, in both boutons and primary/secondary branches of the exciter axon was monitored after iontophoresis of the ratiometric calcium dye fura-2. Resting $[Ca^{2+}]_i$ in boutons was measured as 169 ± 42 nM (n = 4). Tetanic stimulation at 20 Hz resulted in calcium accumulation to a concentration of 664 ± 114 nM at release sites and throughout the axonal arbor (Figure 4A). On termination of the tetanus, $[Ca^{2+}]_i$ decayed back to pre-tetanic levels within minutes, demonstrating that EJP amplitude increase was not associated with any detectable increase in residual calcium during the time course of LTF expression. This confirms the findings of Delaney et al. (1989).

Even though $[Ca^{2+}]_i$ was not elevated from pretetanus levels during LTF expression, it remained a possibility that calcium influx during the tetanus initiated LTF. To test this hypothesis, we delivered the LTF-inducing tetanus in the absence of external calcium, to prevent calcium entry and thus reduce tetanic calcium accumulation. Two different media were used to achieve this: introduction of a Mg^{2+}-substituted Ca^{2+}-free Van Har-

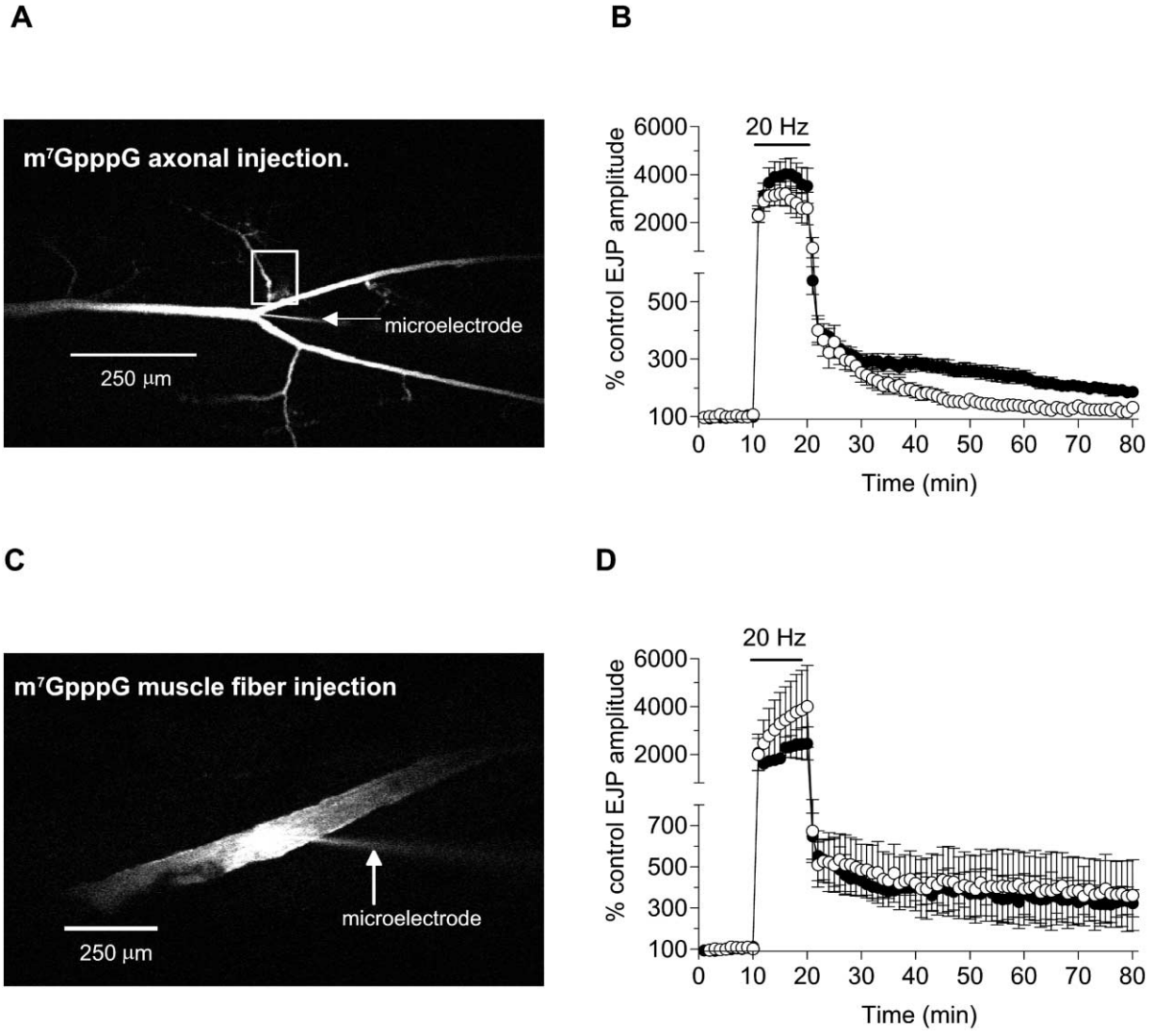

Figure 3. Presynaptic, but Not Postsynaptic, Protein Translation Is Required for LTF

(A) Pressure injection of the RNA cap analog m^7GpppG in a fluorescein-marked carrier solution allowed monitoring of axon filling and membrane properties, and estimation of the presynaptic concentration of m^7GpppG (Excitation at 488 nm, 10× objective). Impalement of a proximal muscle cell underlying brightly filled terminals (area indicated by the box) was then achieved using a second electrode.

(B) LTF was significantly impaired after presynaptic injection of m^7GpppG (o) compared to controls (•) recorded from uninjected preparations.

(C and D) Postsynaptic injection of m^7GpppG in fluorescein-marked carrier solution. Recordings from these muscle fibers (o) showed LTF no different from that recorded from muscle cells injected with fluorescein-marked carrier solution alone (•).

revald's solution containing either (1) 6 mM manganese chloride (to block presynaptic calcium channels, Wojtowicz and Atwood, 1988), or (2) 0.75 mM EGTA to chelate the small quantities of calcium ions unavoidably present in this medium. Similar solutions have been used previously to block calcium influx and presynaptic calcium accumulation (Mulkey and Zucker, 1991). Perfusion of either medium did not affect axonal or muscle resting membrane potential, or the integrity of electrophysiological recordings (data not shown), and action potential generation remained unaltered during the tetanus. However, incubation in either medium totally prevented synaptic transmission at 2 Hz, and impaired transmission significantly at 20 Hz, confirming that calcium entry and its accumulation during high frequency stimulation had been substantially blocked (Figures 4B and 4C).

After delivery of the LTF-inducing tetanus in either calcium-free medium, the external solution was rapidly changed to normal Van Harrevald's solution, EJPs recovered, and the amplitude of EJPs during the "LTF expression period" was monitored. Removal of calcium during the tetanus abolished subsequent LTF expression, the increase in EJP amplitude being only 9 ± 24% ($n = 4$) following incubation with 0 Ca^{2+}, 6 mM Mn^{2+}-medium compared to a control increase during LTF of

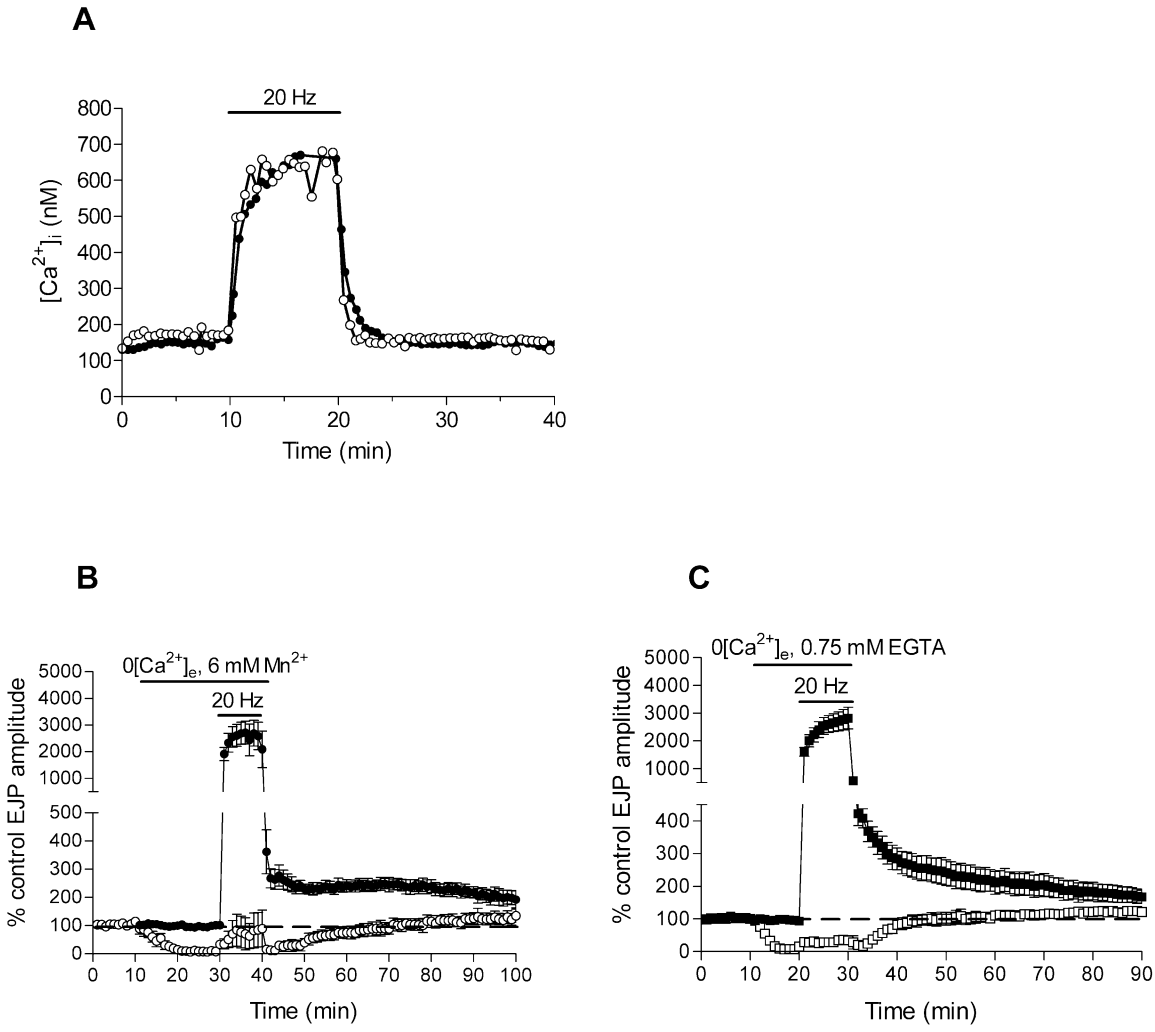

Figure 4. Presynaptic Calcium Entry during the Tetanic Phase Is Required for LTF Induction

(A) Ratiometric measurement of $[Ca^{2+}]_i$ using fura-2 revealed that calcium accumulates in both boutons (o) and primary/secondary branches (•) of the exciter axon in response to a maintained 20 Hz tetanus. By 7 min post-tetanus, resting $[Ca^{2+}]_i$ had fully decayed back to pretetanic resting levels. Data shows the average calcium increase from four separate experiments.

(B) Removal of external calcium and block of calcium channels with Mn^{2+} (6 mM) 20 min prior to and during the 20 Hz tetanus to reduce calcium entry prevented LTF induction (o) as compared to the amplitude of LTF exhibited in normal extracellular solution (•).

(C) Removal of external calcium coupled with external chelation using EGTA (0.75 mM) 10 min prior to and during the 20 Hz tetanus to reduce calcium entry eliminated LTF (□) as compared to the amplitude of LTF exhibited in normal extracellular solution (■).

131 ± 23% (n = 5, $p < 0.005$), and 16 ± 12% (n = 6) in 0 Ca^{2+}, 0.75 mM EGTA-medium versus a control increase during LTF of 100 ± 32% (n = 7, $p < 0.05$).

In conclusion, our results suggest that tetanic presynaptic calcium accumulation is a necessary component of LTF induction. This is only partially in agreement with previous observations (Wojtowicz and Atwood, 1988); we comment further on the discrepancy in the Discussion.

Classical Calcium-Dependent Kinase Pathways Are Not Involved in LTF

As our results implicate the necessity for calcium accumulation during the tetanus, we investigated the involvement of calcium-dependent protein kinases in LTF induction. Calcium/calmodulin-dependent kinase II (CaMKII) inhibition by KN62 and KN93, or protein kinase C (PKC) inhibition with H-7 and myristoylated PKC inhibitor 19-27 (*myr*-PKCI) did not affect LTF (Table 1). Moreover, block of PKA with KT5720 and *Rp*-8-Br-cAMPS, or addition of the relatively nonspecific protein kinase inhibitor K252a (which blocks CaMKII, PKA, PKC, protein kinase G (PKG), myosin light chain kinase (MLCK), and tyrosine kinase) were ineffective against LTF expression (Table 1). Where possible, the efficacy of these inhibitors at the crayfish neuromuscular junction was first established. *Rp*-8-Br-cAMPS penetrates motor nerve terminals and activates I_h channels (Beaumont and Zucker, 2000). PKC-dependent synaptic enhancement by phorbol ester (1 μM) in this preparation is completely pre-

Table 1. Effects of Protein Kinase Inhibitors against LTF

EJP Amplitude Enhancement (%) during LTF			
[Drug]	Kinase Inhibited	Control	+ Inhibtor
KT5720; 1 μM	PKA	83 ± 16 (6)	70 ± 25 (6)
Rp-8Br cAMPS; 300 μM	PKA	124 ± 32 (8)	155 ± 25 (4)
H7; 30 μM	PKC, PKA, PKG	124 ± 32 (8)	145 ± 79 (4)
*myr*PKCI 19–27; 40 μM	PKC	146 ± 24 (4)	115 ± 29 (4)
KN62; 10 μM	CaMKII	50 ± 17 (8)	32 ± 21 (4)
KN93; 10 μM	CaMKII	145 ± 24 (6)	106 ± 21 (3)
SB203580; 6 μM	p38 MAP kinase	97 ± 17 (7)	81 ± 14 (4)
K-252a; 1 μM	Tyrosine PK, CaMKII, PKA, PKC, PKG, MLCK	69 ± 10 (16)	71 ± 16 (3)
PD098059; 30 μM	**MEK (MAPKK)**	**97 ± 17 (7)**	**1 ± 10 (5)[a]**
Wortmannin; 100 nM	**PI3K**	**97 ± 17 (7)**	**12 ± 5 (4)[a]**
LY294002; 15 μM	**PI3K**	**97 ± 17 (7)**	**12 ± 4 (3)[a]**
Rapamycin; 100 nM	**mTOR/FRAP**	**71 ± 16 (8)**	**13 ± 6 (8)[b]**

Data mean ± SEM; numbers of measurements in parentheses; significant changes assessed by Student's unpaired *t* test (shown in bold face); [a]$p < 0.01$, [b]$p < 0.05$.
LTF during kinase inhibition was tested against LTF from the same batch of crayfish (control).

vented by preincubation with H-7 (30 μM) or by *myr*-PKCI (40 μM) (n = 3 and n = 4, respectively, data not shown). PKA and CAMKII have not been shown to mediate any effects in this preparation, and thus a positive control is not possible. However, H-7 should be equally efficacious against both PKC and PKA at the concentration used.

Calcineurin Is Required for LTF Induction

The absence of any obvious calcium-dependent kinase mediation of LTF led us to consider the possibility that the calcium-dependent phosphatase calcineurin (PP2B) may be involved instead. Calcineurin activity has been shown to be essential for the induction of activity-dependent long-term depression in mammals (LTD), which arises from a preferential activation of a phosphatase cascade rather than a kinase cascade in response to modest, prolonged elevations in $[Ca^{2+}]_i$ (Mulkey et al., 1994; Yang et al., 1999). We exposed preparations to a 30 min preincubation and incubation throughout the experimental protocol with either of the specific cell–permeable calcineurin inhibitors cyclosporine (3 μM) or the FK506 analog ascomycin (300 nM). These drugs, which each inhibit calcineurin by first forming complexes with the endogenous immunophillins cyclophilin and FK506BP, respectively, both abolished LTF, with a 0.5 ± 21% (n = 4) and 13 ± 15% (n = 4) increase in EJP amplitude (Figure 5A) compared to a control LTF increase of 82 ± 26% (n = 6). In separate control experiments, we confirmed that application of both cyclosporine (3 μM) and ascomycin (300 nM) together for 90 min at 2 Hz did not affect EJP amplitude (4 ± 9% increase in EJP amplitude, n = 4) recorded at a 50–90 min post-tetanic time point. Furthermore, presynaptic injection of a calcineurin autoinhibitory peptide significantly reduced LTF to an average 25 ± 22% enhancement of EJP amplitude (n = 4), compared to a control enhancement of 97 ± 17% (n = 7). In injected preparations, the level of LTF observed in each preparation was inversely related to the intracellular peptide concentration achieved (ranging from 50–230 μM, with concentrations over 100 μM resulting in complete abolition of LTF; n = 2). These data confirm the presynaptic locus of calcineurin activity (Figure 5A).

Does the requirement for calcineurin activation coincide with the increase in presynaptic calcium? This would imply that calcineurin becomes activated in response to the tetanic calcium rise, and thus is necessary for induction of LTF. To determine this, we applied cyclosporine (3 μM) 5 min prior to, during, and for 5 min following the LTF-inducing tetanus, when presynaptic calcium elevations are apparent (see Figure 4). Incubation with cyclosporine for this limited time was still sufficient to block subsequent expression of LTF (EJP enhancement during LTF following cyclosporine treatment was −6 ± 6% (n = 4), compared to control LTF of 82 ± 26 % ($p < 0.01$; n = 6; Figure 5B)). Conversely, if cyclosporine was added 5 min after the LTF-inducing tetanus and throughout the remaining experimental protocol, LTF expression appeared unchanged from controls, giving a 94 ± 50% EJP amplitude increase (n = 6) compared to 82 ± 26% increase for controls (n = 6; $p > 0.5$; Figure 5C). These data confirm that calcineurin activity is correlated with presynaptic calcium accumulation, and furthermore is required for the induction, but not the expression, of LTF.

LTF Is Blocked by Both PI3K and MAP Kinase Inhibition, and Is Rapamycin Sensitive

The regulation of local protein translation confers an efficient means to rapidly alter protein composition, and hence the potential activity, of synapses. In studies of translational regulation primarily involved in cell development and growth, several kinase pathways have been implicated as key mechanisms governing translation initiation (reviewed by Dufner and Thomas, 1999; Gingras et al., 1999b; and Gray and Wickens, 1998). These kinases are responsible for the phosphorylation of specific eukaryotic initiation factors (most notably eIF4E, 4E-BPs, eIF2, and eIF2B) or ribosomal proteins (such as the S6 subunit of the 40S ribosome). Their phosphorylation status affects either global protein translation rates or the upregulation in translation of specific subsets of mRNA, such as those containing highly structured 5′

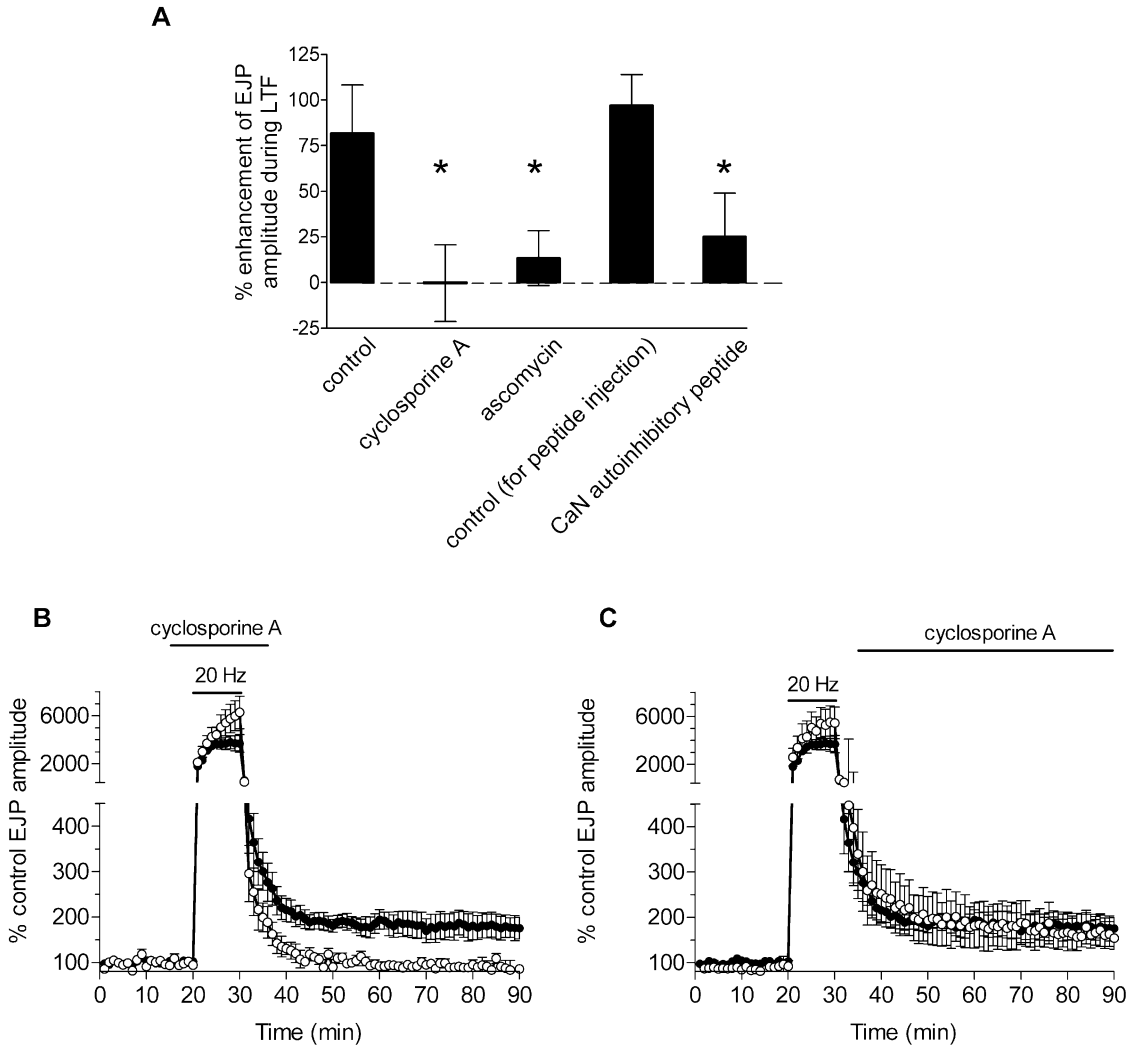

Figure 5. Calcineurin Activation Is Required for LTF Induction, but Not Maintenance, of LTF

(A) Inhibition of calcineurin activity by cyclosporine A (3 μM), ascomycin (300 nM), or presynaptic injection of calcineurin autoinhibitory peptide (50–230 μM) reduced LTF compared to matched controls.

(B) Incubation with cyclosporine 5 min prior to, throughout, and for 5 min following tetanic stimulation was sufficient to prevent LTF induction (ω) compared to controls (•).

(C) Cyclosporine applied following tetanic stimulation did not affect LTF expression (ω) compared to control (•).

noncoding regions or mRNAs with oligopyrimidine tracts in their 5′UTRs (5′TOPmRNAs).

In order to initially determine whether protein phosphorylation played any role in LTF, we used the PP1/PP2A phosphatase inhibitor, calyculin A (1 μM), to prevent the dephosphorylation of proteins whose phosphorylation status may be increased during LTF. Incubation of neuromuscular junctions in calyculin A during basal stimulation (2 Hz) resulted in a small, relatively variable increase in synaptic transmission (37 ± 30% enhancement of EJP amplitude, n = 4), which mostly stabilized following 20–30 min of drug application (Figure 6A). However, when this new level of transmission was taken as the baseline, an LTF-inducing tetanus presented in the presence of calyculin A resulted in a profound potentiation (~14-fold) of LTF expression (881 ± 345% enhancement, n = 5) compared to a control enhancement during LTF of 82 ± 26% (n = 6; Figure 6B). The calyculin-induced potentiation was specific to LTF (rather than extending a PTP phase or affecting other short-term plasticity events), as it could be abolished by coincubation with the calcineurin blocker cyclosporine (Figure 6B), which prevents LTF induction. The resultant LTF was only 57 ± 19% (n = 2), not significantly different from the remaining slow elevation of EJP amplitude which occurred at the same time points during calyculin application alone (24 ± 36%, n = 4). These data provide evidence that phosphorylation of unknown target proteins during LTF plays a key regulatory role or roles.

Several kinases have been identified as regulators of protein synthesis. Most ubiquitous among these are PI3K, the *m*ammalian *t*arget *o*f *r*apamycin kinase (mTOR/FRAP), the 40S ribosomal protein S6 kinase (S6K1), and both p38 and p42/44 MAP kinases, which act via both separate and overlapping pathways to affect the phosphorylation status of components of the translational

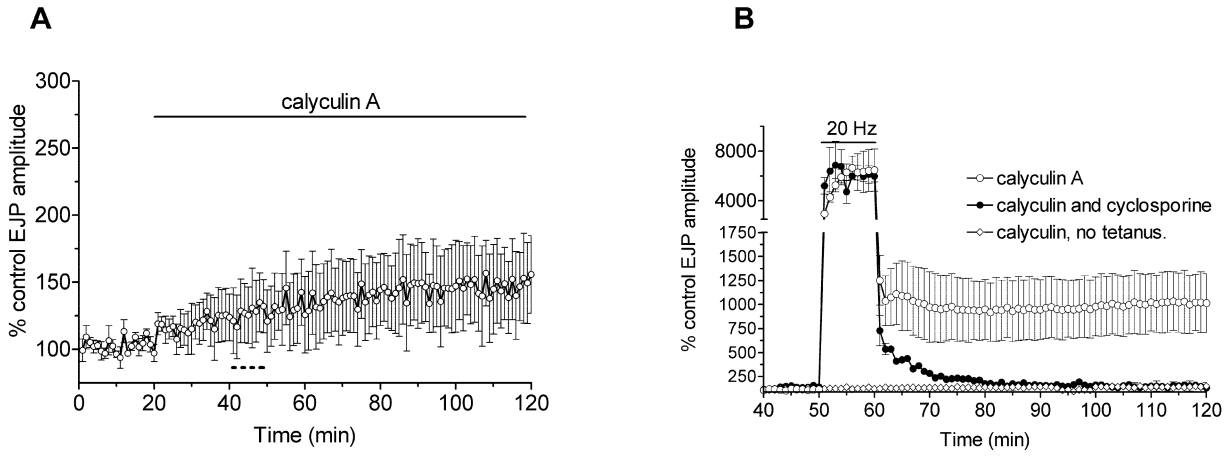

Figure 6. Prevention of Calcium-Independent Dephosphorylation by PP1/PP2A Inhibition Results in a Selective and Profound Potentiation of LTF

(A) Application of calcyculin A (1 μM) during basal 2 Hz stimulation resulted in a small, variable increase in synaptic transmission, which had mostly stabilized after 20–30 min. The dotted line indicates the time period used to renormalize the baseline for subsequent experiments shown in (B).

(B) After normalization of baseline amplitude following 30 min of calyculin (1 μM), subsequent induction of LTF resulted in a massive potentiation of synaptic transmission during LTF in the continued presence of calyculin (o). The potentiation of LTF by calyculin was completely prevented if calcineurin was also blocked by coapplication of cyclosporine (3 μM), to prevent LTF induction (•). EJP amplitude enhancement at 2 Hz stimulation in the presence of calyculin, following renormalization after 20–30 min of calyculin application, is also depicted (◇).

machinery. Using selective pharmacological inhibitors, we investigated the potential role of these kinases in LTF.

Inhibition of MAP kinase/ERK using the specific inhibitor of the upstream MEK (MAP kinase kinase) by PD098059 totally abolished LTF enhancement as compared to controls (Figure 7A, see also Table 1). Conversely, inhibition of the stress-activated P38 MAP kinase with SB203580 did not affect LTF (Figure 7B, Table 1). In addition to the sensitivity to the MAP kinase inhibitor, incubation with either of the PI3K inhibitors wortmannin (Figure 7C) or LY294002 (Figure 7D) profoundly reduced LTF (Table 1). LTF also appeared to be sensitive to the mTOR/FRAP kinase inhibitor rapamycin (Figure 7E).

There is strong precedent in the literature for several alternate MAP kinase, PI3K- and rapamycin-sensitive pathways in the initiation of translation (see Discussion), and thus these data lend strong circumstantial support to the likelihood that these kinases are responsible for the initiation of protein translation during LTF. The novel finding in the present study that in turn, these kinases may be regulated upstream by calcineurin provides a potential point of control that may be pertinent to other forms of activity-dependent protein synthesis.

Discussion

This study provides evidence of a requirement for local presynaptic protein translation in long-term activity-dependent synaptic plasticity. The observation that injection of the cap analog m^7GpppG, which titers out the ability of capped RNA to bind to eIF4E and initiate translation, provides evidence that translation occurs close to release sites (within the area of m^7GpppG diffusion), implying that the mRNA translated is already present in presynaptic terminals or neighboring axon branches.

Mammalian late phase long-term potentiation (L-LTP) is undoubtedly one of the most intensively studied forms of protein synthesis-dependent synaptic plasticity. Convincing evidence suggests that in this case, the regulation of protein synthesis occurs primarily postsynaptically (Nayak et al., 1998) at the level of mRNA transcription, resulting from the PKA-, PKG-, and MAP kinase-dependent phosphorylation of the transcription factor cAMP response element binding protein (CREB) (Huang et al., 2000; Lu et al., 1999; Nguyen and Kandel, 1996, 1997).

However, in invertebrate models of synaptic plasticity, protein synthesis has been shown to be under regulation at both transcriptional *and* translational stages (Casadio et al., 1999; Ghirardi et al., 1995; Martin et al., 1997; Sherff and Carew, 1999), or purely under translational control (Crow et al., 1999; Schilhab and Christoffersen, 1996; Sigrist et al., 2000), allowing rapid but long-term site-specific alterations in synaptic strength. Furthermore, where investigated, protein synthesis has been observed to occur at both postsynaptic (Sigrist et al., 2000) and presynaptic sites in invertebrates (Khan et al., 2001; Martin et al., 1997; Sherff and Carew, 1999). These and our own findings are complemented by ultrastructural evidence demonstrating that ribosomes are present at intermittent intervals along invertebrate axons (Alvarez et al., 2000) and in presynaptic terminals (Crispino et al., 1997; Martin et al., 1998; Sotelo et al., 1999), strongly supporting the view that a local system of protein synthesis is present at presynaptic specializations. It should be mentioned that some of these invertebrate neurons lack the strict polarity of vertebrate neurons, containing both transmissive and receptive surfaces within a single neuritic process (Martin et al., 2000). Protein synthesis-dependent synaptic strengthening in *Aplysia* is largely mediated through (Casadio et al., 1999; Ghirardi et al., 1995; Martin et al., 1997; Sherff and Carew, 1999), or to some degree dependent on (Sutton and Carew, 2000), activation of serotonin receptors on

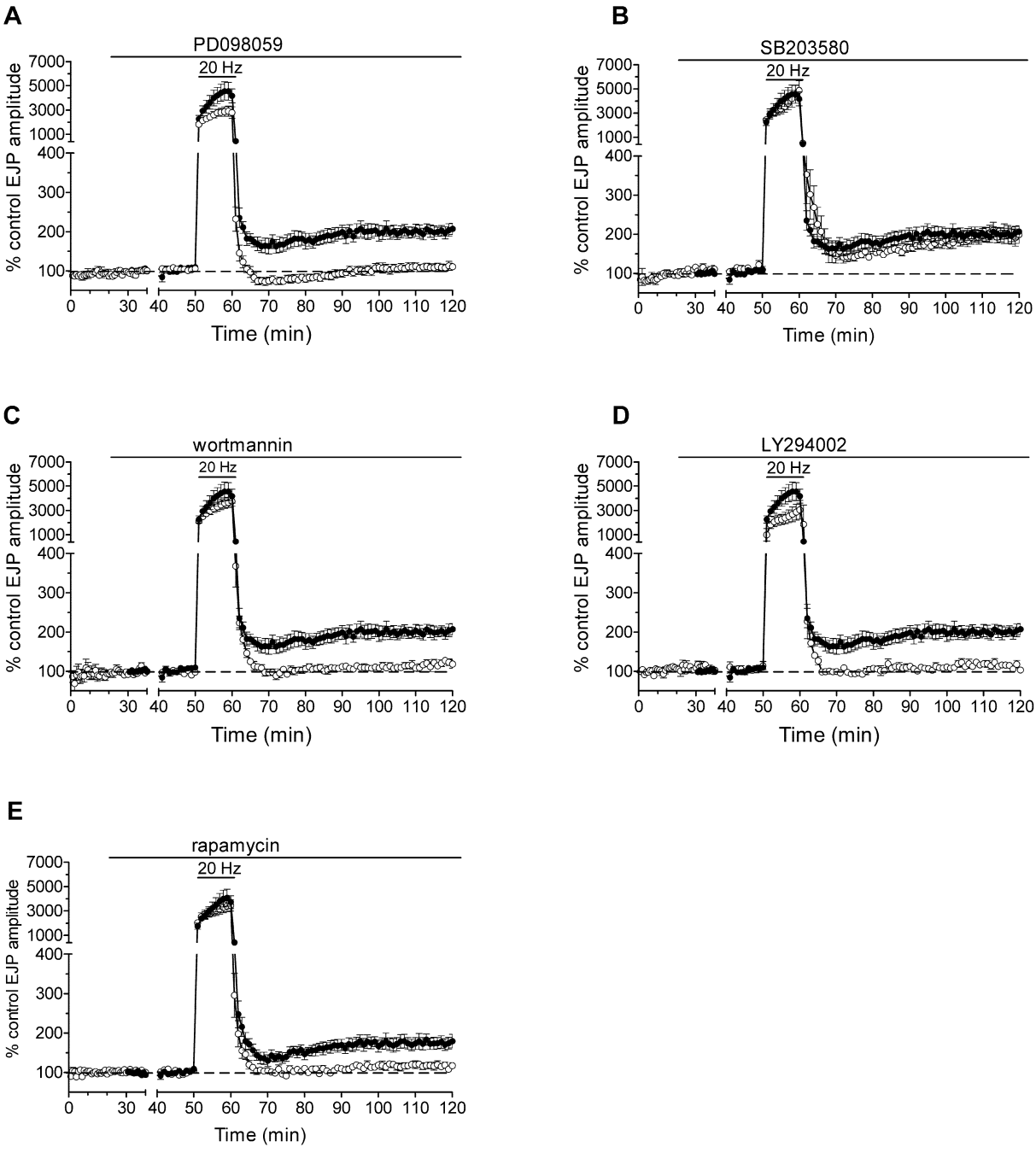

Figure 7. Inhibition of either MAP Kinase, PI3 Kinase, or Rapamycin Is sufficient to Abolish LTF

(A) MEK inhibition by PD098059 (30 μM) to block downstream MAP kinase abolishes LTF (o) compared to controls (•).

(B) Block of the stress-activated P38 MAP kinase by SB203580 (6 μM; o) had no effect on LTF compared to controls (•).

(C–D) Incubation with either of the specific PI3K inhibitors wortmannin (100 nM) or LY294002 (15 μM) was also sufficient to abolish LTF (o) compared to controls (•).

(E) LTF was also sensitive to incubation with rapamycin (100 nM) to block mTOR/FRAP kinase activity (o) as compared to controls (•).

presynaptic sites, which are functionally postsynaptic with respect to endogenous serotonin release.

This does not appear to be the case during crayfish LTF, where LTF induction appears to arise through a calcium-dependent activation of calcineurin, apparently independent of neurohormone/neurotransmitter activation of presynaptic receptors. In theory, the possibility exists that glutamate released from presynaptic terminals during tetanic stimulation may activate presynaptic metabotropic glutamate receptors, but activation of these receptors has been shown previously to exert an inhibitory effect on transmission (Parnas et al., 1996). PI3K and MAPK are kinases that have been repeatedly implicated in neurotrophin signaling (Kaplan and Miller,

2000). The observation that these kinases are necessary for LTF expression raised the possibility that LTF may be neurotrophin mediated, given that neurotrophins can be secreted in an activity-dependent manner from postsynaptic muscle cells (Wang and Poo, 1997; Xie et al., 1997). This possibility was tested using the broad-spectrum protein kinase inhibitor K252a, which is a potent blocker of tyrosine kinase activity associated with a number of neurotrophin receptors (Lazarovici et al., 1996). At 1 μM, this drug had no effect on LTF expression (Table 1), rendering the involvement in LTF of neurotrophins or tyrosine kinase highly unlikely.

Translational control of protein synthesis resulting in rapid synaptic plasticity does not appear to be restricted to the invertebrate nervous system. In forms of mammalian synaptic plasticity other than L-LTP, such as synaptic strengthening by neurotrophins, translational machinery is implicated as the downstream target of neurotrophin action (Bradley and Sporns, 1999; Kang and Schuman, 1996). Local dendritic protein synthesis is required for metabotropic glutamate receptor-mediated long-term depression in the hippocampus (Huber et al., 2000), induction of epileptiform discharges in hippocampus CA3 region (Merlin et al., 1998), and the facilitation of LTP persistence by metabotropic glutamate receptor agonists in hippocampus CA1 region (Raymond et al., 2000).

Mechanistic Insights into LTF Induction

We have presented evidence to suggest that the accumulation of calcium during high frequency stimulation is necessary for LTF induction. This finding is somewhat controversial. While our work is in agreement with previous results showing that delivery of an LTF-inducing tetanus in a 0-Ca^{2+}, EGTA-containing medium prevents LTF, the same study reported that the same experiment performed in a 0-Ca^{2+}, 6 mM Mn^{2+}-containing medium had no effect on subsequent LTF expression (Wojtowicz and Atwood, 1988). We have no explanation for this contradiction. One important caveat that should be mentioned is that the calcium-free medium, which we use to prevent calcium accumulation, also prevents transmitter release. We therefore cannot definitely rule out the inhibition of transmitter release as the cause of LTF prevention, rather than the reduction in presynaptic calcium accumulation. However, the two findings that calcium influx must occur during a tetanus, and that calcium-activated protein phosphatase calcineurin must remain active during this period, lend considerable support for a calcium-dependent regulation of LTF induction via calcineurin activation.

In this respect, LTF induction resembles the induction of mammalian long-term depression (LTD). Calcium influx and calcineurin activation have previously been implicated in LTD induction in both the hippocampus (Mulkey et al., 1994; Muller et al., 1995; Torii et al., 1995) and visual cortex (Hodgkiss and Kelly, 1995). In the hippocampus, calcineurin activation activates a downstream phosphatase cascade by dephosphorylation of the inhibitory protein to PP1/PP2A activity (Mulkey et al., 1994). This is not the case with LTF, as PP1/PP2A inhibition with calyculin has diametrically opposite effects to calcineurin inhibition, suggesting that instead calcineurin activation may result in a dephosphorylation event leading to activation of a kinase cascade, which is regulated in situ by endogenous PP1/PP2A phosphatase activity. However, the recent finding that NMDAR-dependent LTD (the calcium/calcineurin-dependent form of LTD) is subsequently maintained by de novo protein translation in hippocampal organotypic slices (Kauderer and Kandel, 2000) raises the interesting possibility that both processes may share some common mechanisms.

Although our results clearly indicate the necessity for calcium accumulation and calcineurin activation in LTF induction, we should mention that in addition to these processes, we have discovered the necessity for tetanic presynaptic sodium accumulation and subsequent activation of the electrogenic Na^+/K^+ATPase in LTF induction. This activation leads to membrane hyperpolarization and activation of presynaptic I_h channels during the period in which sodium concentration remains elevated (roughly equivalent to the time of calcium elevation). Block of I_h channels with specific inhibitors results in a profound reduction in LTF expression. The induction of LTF by this process is discussed in detail in a separate paper (V. Beaumont et al., submitted).

Mechanistic Insights into Translational Regulation during LTF

In this and other studies where protein synthesis has been shown to be critical for synaptic strengthening, an unresolved question is whether protein synthesis is necessarily *initiated* during LTF induction. Alternatively, there might be a rapid basal turnover of the protein(s) required for LTF expression, and "induction" involves the *stabilization* of those proteins (presumably via post-translational modifications), rendering them able to affect synaptic excitability or perhaps to become more resistant to protein degradation. We attempted to address this question by manipulation of processes that are key candidates mediating translation initiation. The role of phosphorylation in regulating protein synthesis is well established. The profound potentiation of LTF following inhibition of PP1/PP2A by calyculin suggests that phosphorylation is critical in regulating LTF, perhaps by modulation of a step controlling protein synthesis.

The observation that certain hormones, growth factors, and mitogens promote cell growth and survival led to the identification of several kinases which are activated in response to these external stimuli. Of these, MAP kinase, PI3 kinase, and mTOR/FRAP have been identified as major regulators of the phosphorylation status of translational machinery. For instance, PI3K, operating through rapamycin–sensitive and –insensitive kinase cascades, alters the phosphorylation status of eIF4E binding repressor protein 4E-BP, the P^{70} S6 kinase (S6K1), and the guanine nucleotide exchange factor eIF2B. In the case of 4E-BP, phosphorylation relieves the interaction of 4-EBP with eIF4E, which binds to eIF4E with high affinity in a hypophosphorylated state, preventing eIF4E interaction with eIF4G and the formation of the active eIF4F multiprotein cap binding complex (Gingras et al., 1999a, 1999b; reviewed by Raught and Gingras, 1999). This PI3K-dependent pathway involves signaling by PKB/Akt as well as FRAP/ mTOR, and the implication of both PI3K and mTOR activation during

LTF highlights 4E-BPs as potential targets for translational regulation at crayfish presynaptic sites.

PI3K also phosphorylates and activates S6K1 (for review, see Dufner and Thomas, 1999), which is also under a parallel regulation by mTOR/FRAP. Activation of S6K1 is thought to phosphorylate the S6 subunit of ribosomal 40S protein, leading to a preferential upregulation in the translation of 5′TOPmRNAs (Amaldi and Pierandrei-Amaldi, 1997). A recent report has implicated S6 kinase activation via a rapamycin-sensitive pathway following serotonin application in *Aplysia* synaptosomes (Khan et al., 2001). While MAPK has also been shown to phosphorylate S6K1 in vitro, studies employing dominant interfering mutants of the Ras/MAPK pathway demonstrated that MAPK is neither necessary nor sufficient for S6K1 activation (Ming et al., 1994). Instead, one of the major targets of MAPK/ERK (and p38 MAP kinase) in translational control appears to be the phosphorylation of eIF4E (see reviews by Gingras et al., 1999b; Raught and Gingras, 1999), which is thought to result in a stronger affinity of eIF4E for capped RNA.

More work is needed to elucidate the translational control involved in crayfish LTF. However, the finding that either PI3K, mTOR, or MAPK inhibition alone is sufficient to prevent LTF suggests that translational regulation is likely to be a complex, multiply regulated process involving a number of converging kinase pathways, acting synergistically to regulate translation.

What proteins are synthesized during synaptic plasticity? In the case of crustacean LTF, the answer will probably require identification of the specific mRNA localized to presynaptic terminals or neighboring axonal sites. This approach has been used in neuronal dendrites (and Schuman, 1999; reviewed by Steward, 1997) and has already shed light on some of the proteins upregulated during L-LTP (Guzowski et al., 2000; Nayak et al., 1998; Roberts et al., 1998). Previous ultrastructural data suggesting that crayfish LTF is associated with an increase in number of active zones per synapse (Wojtowicz et al., 1994) allow cautious speculation that perhaps components of the transmitter release apparatus are synthesized, but this hypothesis remains to be tested.

Experimental Procedures

Crayfish (*Procambarus clarkii*, 2.0–3.5 in long) were obtained from Niles Biological (Sacramento, CA) and KLM Bioscientific (San Diego, CA). Preparation of the innervated dactyl opener muscle of the first walking leg has previously been described (Delaney et al., 1991). Autotomized legs were maintained at 14°C–17°C and continuously superfused by a gravity-fed perfusion system with Van Harrevald's solution, containing (in mM) NaCl 195, $CaCl_2$ 13.5, KCl 5.4, $MgCl_2$ 2.6, and Na-HEPES 10 (pH 7.4). H-7, PD098059 and cycloheximide were obtained from Sigma (St Louis, MO). RNA cap analog m^7(5′)Gppp(5′)G was obtained from Amersham (Arlington Heights, IL), and SB203580 and K252a were obtained from A.G. Scientific Inc. (San Diego, CA). All other drugs were from Calbiochem (La Jolla, CA). When stock solutions of drugs had to be dissolved in DMSO or ethanol, the final concentration of solvent remained below 0.1%.

Electrophysiology

Sharp electrodes were used to impale both proximal muscle fibers (electrode resistance 12–25 MΩ) and/or either primary or secondary branches of the exciter nerve axon (beveled electrode resistance 25–45 MΩ). Recordings of membrane properties were made during exciter nerve stimulation using a suction electrode containing the axon freed from the meropodite segment of the leg. Electrical signals were amplified (Neuroprobe 1600 Amplifier, A-M Systems, Everett, WA), filtered at 2 kHz, digitized at 5 kHz, and the average of all EJPs elicited each minute saved to computer using pClamp7 software (Axon Instruments, Foster City, CA). EJP amplitudes were measured offline (Clampfit 6.05, Axon Instruments).

Data Presentation and Statistical Analysis

Results have been expressed as the percent change from control EJP amplitude, taken as the average EJP amplitude acquired from 10–20 min continuous recording at 2 Hz. Data are plotted as the mean ± SEM percent change from this control level. As "control" LTF and LTF in the presence of various drugs were obtained in different preparations, statistical analysis of results was achieved by Student's unpaired t test and significance assumed with $p < 0.05$. When LTF in the presence of drugs was compared to control LTF, either graphically or quantitatively in the text, this was done against controls obtained from the same batch of crayfish, to avoid erroneous interpretation of drug effects that may have occurred due to seasonal, batch, or other variations.

Presynaptic Injection Procedures

Pressure injection of impermeable compounds m^7GpppG (2.5 mM) or calcineurin autoinhibitory peptide (αα457-482; *H*-ITSFEEAKGL DRINERMPPRRDAMP-*OH;* 1 mM) in a dye-marked carrier solution (0.5 mM fluorescein, 200 mM KCl, 10 mM HEPES, pH 7.4) was accomplished by positive pressure (20–40 psi, 1 s pulses) applied to the lumen of a microelectrode, until fluorescein could be detected (at 480 nm illumination) throughout the axonal arbor (normally after 10–30 min). The intensity of fluorescence in the axon was measured, and this value was used to estimate the intracellular concentration of fluorescein and, by extrapolation, the compounds of interest, according to methods previously described (Delaney et al., 1989).

Measurement of Intracellular Calcium

The exciter axon was penetrated near the Y-branch with a microelectrode filled with fura-2 (17 mM in 200 mM KCl). Filling of the axon was achieved by iontophoresis of dye by continuous hyperpolarizing current (5–15 nA, 15 min). The final concentration of fura-2 in the axon was estimated at 150 μM. Fluorescence was detected with a silicon intensified target (SIT) camera (model 66, Dage MTI, Michigan City, IN), via a 40× Olympus water immersion objective (Lake Success, NY). Fluorescence was alternately excited through 350 ± 10 nm and 382 ± 5 nm filters (Omega Optics, Battleboro, VT). A dichroic mirror (455 nm, Nikon, Japan) separated excitation and emission lights, and a barrier filter (530 ± 20 nm, Omega Optics) limited interference by autofluorescence. An area near the imaged axon with uniform intensity similar to that around the axon was chosen to obtain a background subtraction level. Shading correction was taken from a 50 μm path-length cuvette containing 50 μM fura-2 in a nominally Ca^{2+}-free calibration solution (see calibration procedures). Background subtraction and shading correction were performed at the start of each experiment and subsequent images generated automatically from the calibration parameters on an image processor (Gould FD 5000, Fremont, CA). Averages of 32 sequential 350 nm- and 380 nm-excited images were stored on an optical disk recorder (Panasonic, TQ-208F, Secaucas, NJ). The imaging processor, optical disk recorder, and filter changer were under the control of a Scientific Microsystems SMS 1000 computer (Mountain View, CA) using software written by Dr. Roger Tsien (University of California, San Diego, CA).

Calibration Procedures

Fura-2 (50 μM) was calibrated in vitro by measuring the fluorescence ratio in solutions resembling crayfish axoplasm (in mM): K-gluconate 250, NaCl 15, K-HEPES 15, pH 7.02 with zero-calcium (K_2EGTA 10), 5 mM Ca^{2+} or Ca^{2+} buffered to 500 nM with 10 mM K_2EGTA and 5 mM $CaCl_2$. Using Equation 5 of Grynkiewicz et al. (1985), the K_d of Fura-2 for calcium was estimated as 469 ± 38 nM (n = 5). A 30% viscosity correction was applied to compensate the cuvette fluorescence measurements for effects of crayfish axoplasm on in situ fluorescence (Delaney et al., 1989).

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