Forskolin induction of late-LTP and up-regulation of 5' TOP mRNAs translation via mTOR, ERK, and PI3K in hippocampal pyramidal cells

Delphine Gobert^{*}, Lisa Topolnik^{*,1}, Mounia Azzi^{*,2}, Linda Huang[†], Frédérique Badeaux[†], Luc DesGroseillers[†], Wayne S. Sossin[‡], and Jean-Claude Lacaille^{*}

^{*}Département de Physiologie, Groupe de Recherche sur le Système Nerveux Central, Université de Montréal, Montréal, QC, Canada

[†]Département de Biochimie, Université de Montréal, Montréal, QC, Canada

[‡]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montréal, QC, Canada

Abstract

The late phase of long-term potentiation (LTP) requires activation of the mammalian target of rapamycin (mTOR) pathway and synthesis of new proteins. mTOR regulates protein synthesis via phosphorylation of 4E-binding proteins (4E-BPs) and S6K, and via selective up-regulation of 5' terminal oligopyrimidine (5' TOP) mRNAs that encode components of the translational machinery. In this study, we explored the regulation of 5' TOP mRNAs during late-LTP (L-LTP). Synaptic plasticity was studied at Schaffer collateral – CA1 pyramidal cell synapses in rat organotypic hippocampal slices. Forskolin, an adenylate cyclase activator, induced L-LTP in organotypic slices that was mTOR-dependent. To determine if 5' TOP mRNAs are specifically up-regulated during L-LTP, we generated a 5' TOP-myr-dYFP reporter to selectively monitor 5' TOP translation. Confocal imaging experiments in cultured slices revealed an increase in somatic and dendritic fluorescence after forskolin treatment. This up-regulation was dependent on an intact TOP sequence and was mTOR, extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)-dependent. Our findings indicate that forskolin induces L-LTP in hippocampal neurons and up-regulates 5' TOP mRNAs translation via mTOR, suggesting that up-regulation of the translational machinery is a candidate mechanism for the stabilization of LTP.

Keywords

5' terminal oligopyrimidine tract mRNAs; mammalian target of rapamycin; synaptic plasticity; translation

Address correspondence and reprint requests to Jean-Claude Lacaille, Département de Physiologie, Groupe de Recherche sur le Système Nerveux Central, Université de Montréal, C.P. 6128, succursale Centre-ville, Montréal, QC H3C 3J7, Canada. jean-claude.lacaille@umontreal.ca.

¹The present address of Lisa Topolnik is the Département de Biochimie et de Microbiologie, Centre de Recherche Université Laval Robert-Giffard, Unité de Neurobiologie Cellulaire, 2601 Chemin de la Canardière, Beauport, QC G1J 2G3, Canada.

²The present address of Mounia Azzi is Neurochem Inc., 275 Armand-Frappier Blvd, Laval, QC H7V 4A7, Canada.

Long-term potentiation (LTP), a candidate for the cellular basis of memory, requires a complex cascade of molecular and cellular events. Classically, LTP can be divided into two stages: an early stage that depends on phosphorylation and modification of pre-existing proteins (early-LTP, lasting minutes to hours) and a late stage that requires transcription and translation of new proteins [late-LTP (L-LTP), lasting several hours]. While protein synthesis can take place both in the cell body and the dendrites of hippocampal neurons, local protein synthesis is thought to play an important role for the reinforcement of specific synaptic connections during late-phase LTP (Kelleher *et al.* 2004b; Sutton and Schuman 2006). In the hippocampus, cAMP and mammalian target of rapamycin (mTOR) pathways have both been shown to be required for the late-form of LTP (Wong *et al.* 1999; Tang *et al.* 2002; Cammalleri *et al.* 2003; Wang *et al.* 2004) but it is still unclear whether these two pathways interact with each other or represent independent inputs.

Mammalian target of rapamycin activation regulates protein synthesis via phosphorylation of 4E-BPs and S6K, and via selective translation of 5' terminal oligopyrimidine tract (5' TOP) mRNAs, a subset of mRNAs encoding components of the translational machinery (Gingras *et al.* 2001; Hay and Sonenberg 2004). The 5' TOP mRNA family is characterized by the presence of a 5' TOP and comprises all the ribosomal proteins and several elongation factors (Meyuhas 2000; Meyuhas and Hornstein 2000). Translation of 5' TOP mRNAs is regulated by the status of the cell: they are selectively stimulated during cell growth and specifically repressed upon growth arrest (Meyuhas 2000; Meyuhas and Hornstein 2000).

Recently, it has been suggested that regulating the synthesis of the translational machinery via mTOR in dendrites might play an important role for the maintenance of L-LTP and the reinforcement of specific synaptic connections (Tsokas *et al.* 2005, 2007; Poon *et al.* 2006; Liao *et al.* 2007). However, while the levels of several 5' TOP mRNAs including elongation factor 1A (eEF1 α), S6, eEF2, and poly-A binding protein (PABP) have been shown to be up-regulated during LTP in an mTOR-dependent manner (Tsokas *et al.* 2005, 2007), it is still unknown if this is because of their TOP sequence or other translational regulatory pathways.

In the present study, we investigated the relationships between cAMP and mTOR pathways in the regulation of 5' TOP mRNAs and in L-LTP induced by forskolin, an adenylate cyclase activator. Using fluorescent reporters designed to specifically monitor 5' TOP mRNAs translation, we found that forskolin specifically up-regulates 5' TOP mRNAs translation in dendrites via activation of the mTOR pathway in a TOP-dependent manner. This suggests that increasing the translational capacity of synapses via the selective synthesis of 5' TOP mRNAs, could play an important role for the maintenance of L-LTP.

Materials and methods

Electrophysiology

Transverse hippocampal slices (400- μ m thick) were obtained from male Sprague–Dawley rats (4–6 weeks old) using a vibratome (Leica VT1000S, Leica Microsystems GmbH, Wetzlar, Germany) as described previously (Costa-Mattioli *et al.* 2005). Slices were kept submerged at 26–27°C and superfused (1–2 mL/min) with oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.25

NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, and 10 glucose. A bipolar tungsten stimulating electrode was placed in the CA1 *stratum radiatum* to stimulate the Schaffer collateral and commissural fibers, and extracellular field excitatory post-synaptic potentials (fEPSPs) were recorded with a glass microelectrode (2–3 MΩ, filled with 2 M NaCl) positioned in the *stratum radiatum*. LTP was induced either electrically using repetitive high frequency stimulation (four trains at 100 Hz, delivered 5 min apart) or chemically using forskolin (50 μM, Calbiochem, La Jolla, CA, USA), an adenylate cyclase activator. Whenever indicated, ACSF was supplemented with rapamycin (200 nM, Sigma, St. Louis, MO, USA) or anisomycin (40 μM, Calbiochem) at least 30 min prior to LTP induction.

Organotypic slice culture

Hippocampal slice cultures were obtained from P7 Sprague–Dawley rats as described previously (Stoppini *et al.* 1991; Bourdeau *et al.* 2007). In brief, the brain was removed and dissected in Hanks' Balanced Salt Solution (Invitrogen, Canada, Burlington, ON, Canada)-based medium. Cortico-hippocampal slices (400-μm thick) were then obtained using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd, Gomshall, UK). After dissection, slices were placed on Millicell culture plate inserts (Millipore, Billerica, MA, USA) and allowed to recover in OptiMEM (Invitrogen) kept at 37°C in a humidified atmosphere (95% air, 5% CO₂) for 48 h. They were then maintained in a Neurobasal medium (Invitrogen) supplemented with B27 and Glutamax I (Invitrogen) for 7–10 days. Usually, slices were transfected and processed for experiments after 1 week in culture.

Western blotting

Organotypic slice cultures were prepared as described above and acute transverse hippocampal slices (400-μm thick) were obtained from male Sprague–Dawley rats (4–6 weeks old) using a McIlwain tissue chopper. Slices were allowed to recover for 1 h in slice holding chambers containing heated ACSF (32°C) before receiving the indicated treatment. Slices were then processed for blotting as described previously (Topolnik *et al.* 2006). Briefly, hippocampal extracts were homogenized in ice-cold radioimmunoprecipitation assay buffer containing: 10 mM Tris pH 8, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.0001% Chymostatin, leupeptin, antipain, pepstatin (CLAP) protease inhibitors, 200 μM Na₃VO₄, 200 μM NaF, and 0.2 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 14 000 *g* for 20 min at 4°C to remove debris, and protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Next, equal sample amounts (50 μg/lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk, 0.25% bovine serum albumin dissolved in tris-buffered saline-tween 20 buffer for 1 h at 23°C. After blocking, the membrane was probed with primary antibodies [phospho-S6K Thr389, phospho-S6K Thr421/Ser424, phospho-S6 Ser235/236, and phospho-Akt Ser473, (1 : 1000; Cell Signaling, Beverly, MA, USA) and phospho-extracellular signal-regulated kinases (ERK) Thr202/Tyr204, (1 : 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA)] overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at 23°C. Immunoreactive bands were detected by enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA). The membrane was then

stripped with a buffer containing 0.1 M glycine pH 2.2, 1% sodium dodecyl sulfate and reprobed with antibodies detecting levels of total S6K, S6, Akt, and ERK (Cell Signaling). All immunoreactive bands were scanned with a desktop scanner and quantified with QUANTITY ONE software (Bio-Rad, Hercules, CA, USA).

Constructs

Plasmids encoding pL32 and pL32C-A coupled to green fluorescent protein were a kind gift of Dr. Oded Meyuhas (Tang *et al.* 2001). Destabilized myristoylated versions of enhanced yellow fluorescent protein were inserted into the pL32 and pL32C-A plasmids, replacing enhanced green fluorescent protein, which gave the 5' TOP reporter (5' pL32-myr-dYFP) and the mutated 5' TOP reporter (5' pL32C-A-myr-dYFP), respectively. The sufficient and essential 640 bp (Blichenberg *et al.* 1999) of the dendritic targeting element of the 3'UTR (untranslated region) of microtubule associated protein from *Rattus norvegicus* was inserted in the 5' TOP reporter and the mutated 5' TOP reporter, which gave the 5'-TOP-MAP2-3'UTR reporter (5'-pL32-myr-dYFP-MAP2-3' UTR) and the mutated 5'-TOP-MAP2-3' UTR reporter (5'-pL32C-A-myr-dYFP-MAP2 3' UTR), respectively.

HEK293 cells experiments

Human embryonic kidney cells (HEK293) cells were cultivated in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine (Wisent Canada, QC) at 37°C in a humidified atmosphere (95% air, 5% CO₂). One day prior to transfection, HEK293 cells were plated onto glass coverslips and placed in 35-mm culture dishes. HEK293 cells were transfected by calcium phosphate-DNA precipitation with either the 5' TOP reporter (5'-pL32-myr-dYFP) or the mutated 5' TOP reporter (5'-pL32C-A-myr-dYFP). After 24 h, cells were starved overnight before receiving serum and/or 20 nM rapamycin for 4 h. After this point, cells were quickly washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and processed for fluorescence imaging.

Transfection of hippocampal slice cultures

Gold beads (1.6 µm, Bio-Rad) were coated with 50 µg cDNA of either 5' TOP reporter (5'-pL32-myr-dYFP), mutated 5' TOP reporter (5'-pL32C-A-myr-dYFP), 5'-TOP-MAP2-3' UTR reporter (5'-pL32-myr-dYFP-MAP2-3' UTR), or mutated 5'-TOP-MAP2-3' UTR reporter (5'-pL32C-A-myr-dYFP-MAP2-3' UTR). After 1 week in culture, organotypic slices were transfected using a Helios GeneGun (Bio-Rad) according to the manufacturer's instructions (150 psi at a distance of 1.5 cm). Slices were then returned to the incubator for 48 h before being treated, fixed, and processed for fluorescence imaging.

Confocal microscopy

After treatment, transfected hippocampal slices were fixed overnight in 4% paraformaldehyde. Slices were then quickly washed with phosphate-buffered saline and mounted in Prolong Antifade kit (Molecular Probes, Invitrogen). Images were acquired in 0.4–0.5 µm sections using a confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany). Yellow fluorescent protein was excited at 514 nm and emitted light

was collected using a long-pass filter (cut-off 530 nm). Acquisition parameters and settings were kept constant for all conditions on a given experimental day. Images were analyzed using either LSM 510 (Carl Zeiss) or IMAGEJ software (NIH, Bethesda, MD, USA). Analysis of mean fluorescence levels was conducted on z-compressed stacks that contained the entire neuron or dendrite of interest.

Statistical analysis

All data are presented as means $\pm$ SEM and '*n*' indicates the number of cells or slices. Statistical significance was determined using two-tailed Student's *t*-tests or one-way ANOVAs. Differences were considered significant when $p < 0.05$.

Results

Forskolin induces L-LTP in organotypic hippocampal slices

In acute hippocampal slices, high-frequency stimulation (four trains at 100Hz delivered 5 min apart) of Schaffer collateral/commissural fibers generates a sustained potentiation of fEPSP responses (Fig. 1a and b; at 240 min, $146.2 \pm 17.5\%$ of baseline for vehicle-treated slices, $n = 7$). As previously reported (Tang *et al.* 2002; Cammalleri *et al.* 2003), rapamycin, an inhibitor of the mTOR, selectively inhibited the late-phase of LTP in acute hippocampal slices (at 240 min, $96.4 \pm 9.6\%$ of baseline for rapamycin-treated slices, $n = 6$; $p < 0.05$). However, induction protocols such as four trains of high-frequency stimulation or theta-burst stimulation did not reproducibly induce L-LTP of field responses in hippocampal slice cultures (data not shown; Otmakhov *et al.* 2004; Kopec *et al.* 2006). This may be because of heavy reorganization of Schaffer collaterals in cultured slices leading to difficulty in achieving sufficient post-synaptic depolarization in a large number of cells to trigger the molecular cascades that induce LTP of field responses or because of the loss of modulatory inputs (such as dopaminergic fibers) that may be required to induce L-LTP (Frey *et al.* 1991; Matthies *et al.* 1997). On the other hand, forskolin, an adenylate cyclase activator, could induce a robust and sustained potentiation of fEPSPs responses recorded in CA1 *stratum radiatum* in slice cultures (Fig. 1c and d; at 240 min, $141.9 \pm 9.8\%$ of baseline for forskolin-treated slices, $n = 6$) (Otmakhov *et al.* 2004; Kopec *et al.* 2006). Moreover, forskolin-induced L-LTP was blocked by both anisomycin and rapamycin (at 240 min, $78.6 \pm 9.6\%$ of baseline for anisomycin-treated slices, $n = 3$, $p < 0.01$; $86.4 \pm 9.9\%$ of baseline for rapamycin-treated slices, $n = 7$, $p < 0.01$). Therefore, forskolin-induced L-LTP shares similar properties with L-LTP induced by high-frequency stimulation such as dependency on the synthesis of new proteins and activation of the mTOR pathway.

Forskolin increases the activity of several components of the mTOR pathway

Next, we verified if adenylate cyclase stimulation could indeed activate components of the mTOR pathway in acute slices. Signaling to mTOR pathway is commonly achieved via phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) (Gingras *et al.* 2001; Hay and Sonenberg 2004) but it is not known if cAMP can also activate this pathway. Using western blot analysis, we assessed phosphorylation levels of S6K (Thr389) and S6 (Ser235/236) after forskolin treatment as markers of mTOR activation. Application of forskolin to hippocampal slices markedly increased the phosphorylation of S6K and S6 (Fig.

2a and b for phospho-S6K: $168.3 \pm 15.2\%$, $n = 5$, $p < 0.01$; for phospho-S6: $162.2 \pm 25.1\%$, $n = 12$, $p < 0.05$). This increase was blocked by pre-treatment with rapamycin (for phospho-S6K: $96.5 \pm 17.9\%$, $n = 5$, $p > 0.05$; for phospho-S6: $93.3 \pm 17.8\%$, $n = 12$, $p > 0.05$). In contrast, forskolin treatment-induced phosphorylation of Akt (Ser473) and ERK (Tyr204) (Fig. 2c and d; for phospho-Akt: $151.3 \pm 33.8\%$, $n = 6$, $p < 0.001$; for phospho-ERK: $157.2 \pm 20.8\%$, $n = 4$, $p < 0.001$), but these augmentations were not prevented by the addition of rapamycin (for phospho-Akt: $148.6 \pm 27.8\%$, $n = 6$, $p < 0.001$; for phospho-ERK: $155.8 \pm 16.5\%$, $n = 5$, $p < 0.001$). Both Akt and ERK are upstream of mTOR, thus confirming the specificity of rapamycin. These results demonstrate that forskolin stimulates phosphorylation of S6K and S6 via activation of the mTOR pathway in hippocampal slices.

Forskolin selectively activates a 5' TOP reporter via mTOR in HEK cells

While regulation of 5' TOP mRNAs is usually regulated by mTOR, there are also counterexamples to this paradigm (Tang *et al.* 2001). Moreover, specific regulation by TOPs has not been investigated in neurons previously, and regulatory pathways in neurons are often specialized. To specifically determine if there is regulation of translation of TOP-containing mRNAs during LTP, we generated two different fluorescent reporters based on previous work examining regulation of 5' TOP mRNAs (Avni *et al.* 1994). All 5' TOP mRNAs have a cap structure followed by a C residue and an uninterrupted stretch of 4–14 pyrimidine residues (Meyuhas 2000; Meyuhas and Hornstein 2000). Mutation of the first C residue to an A residue is sufficient to convert a 5' TOP transcript into a non-TOP-regulated transcript (Avni *et al.* 1994). We thus took advantage of this singularity to test the role of the TOP in regulating translation in hippocampal slices. The first construct contains the L32 promoter (required to ensure correct transcriptional initiation at the TOP), the L32 TOP sequence followed by a sequence encoding a destabilized, myristoylated derivative of YFP (5' TOP reporter: 5'-pL32-myr-dYFP). The second construct is identical, but the first nucleotide of the TOP was converted from a C to an A, removing regulation through the TOP (mutated 5' TOP reporter: 5'-pL32C-A-myr-dYFP).

We first verified the biological activity of our constructs in HEK293 cells. It has previously been shown that growth arrest or serum deprivation (starving) selectively represses translation of 5' TOP mRNAs. In contrast, mitogenic stimuli or serum addition preferentially shifts 5' TOP mRNAs to the polyribosomal fraction and leads to active translation (Meyuhas 2000). Thus, we took advantage of this paradigm to test the activity of our fluorescent reporters.

Human embryonic kidney cells were transfected with either the 5' TOP reporter or the mutated 5' TOP reporter. After an overnight starving, serum replenishment to cultures increased the fluorescence levels of the 5' TOP reporter in HEK293 cells, but there was no change in the level of the mutated 5' TOP reporter (Fig. 3, for the 5' TOP reporter, $n = 33$ cells, $p < 0.001$; for the mutated 5' TOP reporter, $n = 33$ cells, $p > 0.05$). Pre-treatment with rapamycin completely blocked the increase in the level of the 5' TOP reporter ($n = 28$ cells, $p > 0.05$) demonstrating that this was because of mTOR-dependent up-regulation of its translation. Surprisingly, rapamycin pre-treatment revealed an increase in the level of the mutated 5' TOP reporter by serum replenishment ($n = 28$ cells, $p < 0.01$). This may be

because of serum activation of other pathways (including possible non-translational pathways), which were suppressed when mTOR was also activated. Nonetheless, the observed rapamycin-sensitive increase in fluorescence levels of the 5' TOP reporter (TOP-specific) after serum stimulation confirmed that this reporter could effectively monitor TOP-regulated translation.

Forskolin increases somatic 5' TOP mRNAs translation via mTOR in slice cultures

We next investigated the effect of cAMP activation on 5' TOP mRNAs translation using these two constructs in organotypic hippocampal slices. Neurons in cultured slices were transfected with either the 5' TOP reporter or the mutated 5' TOP reporter and fluorescence levels were measured in the cell body of transfected CA1 pyramidal cells. Application of forskolin activated both 5' TOP and mutated 5' TOP reporters in slice cultures (Fig. 4a and b 4 h after forskolin application: for 5' TOP reporter, $150.4 \pm 10.9\%$, $n = 14$ cells, $p < 0.01$; for mutated 5' TOP reporter, $128.5 \pm 11.9\%$, $n = 11$ cells, $p < 0.05$). However, pre-treatment with rapamycin selectively prevented increases in 5' TOP-reporter fluorescence levels (Fig. 4c and d for 5' TOP reporter, $115.7 \pm 8.4\%$, $n = 8$ cells, $p > 0.05$; for mutated 5' TOP reporter, $137.5 \pm 6.5\%$, $n = 6$ cells, $p < 0.01$). This suggests that adenylate cyclase activation can up-regulate somatic 5' TOP mRNAs translation in an mTOR-dependent manner. Although cAMP stimulation also stimulates general translation in the cell body (i.e., other translational mechanisms that are not governed by the presence of the TOP in the 5'-UTR), these are not mTOR-dependent.

Forskolin increases dendritic 5' TOP mRNAs translation via mTOR, ERK, and PI3K in slice cultures

To this point, we have shown that cAMP activation up-regulates 5' TOP mRNAs in the cell bodies of CA1 pyramidal cells and that this requires mTOR activation. However, recent reports have suggested the role of dendritic translation for the reinforcement of specific synaptic connections (Kelleher *et al.* 2004b; Sutton and Schuman 2006). Therefore, we next examined whether up-regulation of 5' TOP mRNAs could also be observed in dendrites. To detect fluorescence in dendrites, we added the MAP2-3' UTR region to our constructs. It has previously been shown that this sequence contains signals that direct the MAP2 mRNA to dendritic regions (Blichenberg *et al.* 1999). When cultured slices were transfected with the 5'-TOP-MAP2-3' UTR reporter, fluorescence levels in CA1 pyramidal cell dendrites were markedly increased by forskolin treatment (Fig. 5a-c). This augmentation was significant throughout the dendritic length examined, from the most proximal region (Fig. 5b and c; at 10 μ m from the soma: $209.28 \pm 23.87\%$, $n = 13$ cells for forskolin-treated slices, as compared to $100.0 \pm 9.1\%$, $n = 15$ cells for control slices, $p < 0.001$; normalized to the fluorescence in the first 10 μ m in untreated slices) to the most distal part (at 100 μ m from the soma: $112.8 \pm 21.6\%$, $n = 10$ cells for forskolin-treated slices, as compared to $55.5 \pm 6.6\%$, $n = 10$ cells for control slices, $p < 0.05$). The forskolin-induced increase in fluorescence was prevented by pre-treatment with rapamycin, but only in the most distal region of the dendrite (for rapamycin-treated slices: at 10 μ m from the soma, $163.7 \pm 23.5\%$, $n = 14$ cells, $p < 0.05$; at 100 μ m from the soma, $62.3 \pm 9.2\%$, $n = 13$ cells, $p > 0.05$). In contrast, when neurons were transfected with the mutated 5'-TOP-MAP2-3' UTR reporter, no significant difference was observed in pyramidal neurons for vehicle-treated, forskolin-treated, or forskolin +

rapamycin-treated slices, neither in the proximal dendritic compartment (Fig. 5d–f; at 10 μ m from the soma: $100.0 \pm 8.0\%$, $n = 26$ cells for control slices, $107.3 \pm 16.7\%$, $n = 21$ cells for forskolin-treated slices, $109.6 \pm 21.3\%$, $n = 12$ cells, for forskolin + rapamycin treated slices, $p > 0.05$) nor in the distal dendritic compartment (at 100 μ m from the soma: $49.7 \pm 5.3\%$, $n = 23$ cells for control slices, $41.4 \pm 6.0\%$, $n = 18$ cells for forskolin-treated slices, $39.9 \pm 8.5\%$, $n = 9$ cells for forskolin + rapamycin-treated slices, $p > 0.05$). These data indicate that cAMP stimulation specifically up-regulates 5' TOP mRNAs translation through mTOR in dendrites of hippocampal pyramidal cells.

Nonetheless, differences in the regulation of 5' TOP mRNAs seem to exist between proximal and more distal dendrites of cultured hippocampal pyramidal cells. As described above, we observed that fluorescence in proximal dendrites could not be blocked by a low dose of rapamycin, although the same dose completely prevented the increase in fluorescence in more distal dendrites (Fig. 5a–c). There are several possibilities for this discrepancy, including increased mTOR activity and/or presence of additional regulatory pathways in proximal dendrites. To differentiate between these two possibilities, we performed additional confocal imaging experiments with the 5'-TOP-MAP2-3' UTR reporter using a higher dose of rapamycin as well as mitogen activated protein kinase kinase (MEK)/ERK and PI3K inhibitors (Fig. 5g–i). Under these conditions, fluorescence levels were again markedly increased by forskolin treatment from the most proximal dendritic regions (Fig. 5h and i; at 10 μ m from the soma: $149.4 \pm 10.4\%$, $n = 14$ cells for forskolin-treated slices, as compared to $100.0 \pm 3.2\%$, $n = 24$ cells for control slices, $p < 0.001$; normalized to the fluorescence in the first 10 μ m in untreated slices) to the most distal dendritic regions (at 100 μ m from the soma: $83.2 \pm 7.2\%$, $n = 13$ cells for forskolin-treated slices, as compared to $60.3 \pm 3.2\%$, $n = 23$ cells for control slices, $p < 0.05$). This increase in fluorescence was prevented by the addition of either a high dose of rapamycin (1 μ M), an inhibitor of MEK/ERK (PD98059) or an inhibitor of PI3K (LY294002) in both proximal dendrites (at 10 μ m from the soma: $107.2 \pm 6.8\%$, $n = 19$ cells for forskolin + rapamycin-treated slices, $104.5 \pm 9.8\%$, $n = 15$ cells for forskolin + PD98059-treated slices, $101.1 \pm 10.4\%$, $n = 16$ cells for forskolin + LY294002-treated slices, $p > 0.05$) and distal dendrites (at 100 μ m from the soma: $62.5 \pm 5.2\%$, $n = 19$ cells for forskolin + rapamycin-treated slices, $63.1 \pm 7.0\%$, $n = 14$ cells for forskolin + PD98059-treated slices, $61.4 \pm 5.8\%$, $n = 16$ cells for forskolin + LY294002-treated slices, $p > 0.05$). These data contrast with those obtained previously using a lower dose of rapamycin (Fig. 5a–c), in which proximal fluorescence could not be blocked by the addition of the mTOR inhibitor. Moreover, they indicate that throughout the dendritic length, MEK/ERK and PI3K pathways are required for the forskolin-induced increase in 5' TOP mRNA translation.

Forskolin activates the mTOR pathway in an ERK and PI3K-dependent manner in slice cultures

To date, we have shown that mTOR, MEK/ERK, and PI3K pathways are required for forskolin-induced 5' TOP mRNAs translation. However, it is still unclear if these pathways regulate 5' TOP mRNAs in a parallel or linear fashion. To further investigate the mechanism by which cAMP stimulation activates the mTOR pathway, as well as the interactions between MEK/ERK, PI3K and mTOR pathways, we performed additional western blot

experiments and examined the phosphorylation levels of S6K at both the mTOR-dependent site (Thr389) and the ERK-dependent site (Thr421/Ser424). Moreover, to match the experiments using the TOP reporter, we did these experiments on slice cultures as opposed to the earlier experiments on acute slices. Application of forskolin to organotypic slices strongly increased the phosphorylation of S6K at both Thr389 and Thr421/Ser424 (Fig. 6a and b; for Thr389: $142.5 \pm 12.6\%$, $n = 7$, $p < 0.05$; for Thr421/Ser424: $155.8 \pm 14.1\%$, $n = 5$, $p < 0.05$), as well as phosphorylation of S6 (Fig. 6d; $162.5 \pm 11.8\%$, $n = 6$, $p < 0.01$). This increase was blocked by addition of PD98059, a specific MEK/ERK inhibitor (for S6K Thr389: $109.4 \pm 8.8\%$, $n = 7$, $p > 0.05$; for S6K Thr421/Ser424: $107.5 \pm 8.6\%$, $n = 5$, $p > 0.05$; for S6: $114.8 \pm 19.1\%$, $n = 6$, $p > 0.05$). In contrast, rapamycin pre-treatment blocked the increase in phosphorylation of S6K at Thr389 and S6 (for S6K Thr389: $90.4 \pm 3.5\%$, $n = 7$, $p > 0.05$; for S6: $111.3 \pm 9.9\%$, $n = 6$, $p > 0.05$), and had no effect on the increase in phosphorylation of S6K at Thr421/Ser424 ($154.5 \pm 19.0\%$, $n = 5$, $p < 0.05$). Similarly, addition of LY294002, a specific PI3K inhibitor, prevented the increases in phosphorylation of S6K at Thr389 and S6 (for S6K Thr389: $114.7 \pm 13.1\%$, $n = 7$, $p > 0.05$; for S6: $117.1 \pm 12.6\%$, $n = 6$, $p > 0.05$) but did not block the augmentation in phosphorylation of S6K at Thr421/Ser424 ($160.5 \pm 22.6\%$, $n = 5$, $p < 0.05$). Moreover, ERK phosphorylation was increased by cAMP stimulation (Fig. 6c; $150.8 \pm 13.6\%$, $n = 6$, $p < 0.05$), which was blocked by PD98059 ($102.2 \pm 11.7\%$, $n = 6$, $p > 0.05$), but not by rapamycin ($162.8 \pm 20.2\%$, $n = 6$, $p < 0.05$) or LY294002 incubation ($162.9 \pm 18.6\%$, $n = 6$, $p < 0.05$). Taken together, these results suggest that forskolin stimulates phosphorylation of S6K and S6 in an ERK-dependent and PI3K-dependent manner. Moreover, they indicate that mTOR activation in organotypic slices requires the conjunction of both MEK/ERK and PI3K activation.

Discussion

Our results indicate that forskolin induces L-LTP and up-regulation of 5' TOP mRNAs translation in organotypic hippocampal slices via activation of the mTOR pathway (Fig. 7). First, forskolin, an adenylate cyclase activator, induces a sustained potentiation of fEPSPs that is sensitive to rapamycin treatment. In addition, cAMP activation increases the activity of S6K and S6, two components of the mTOR pathway, in an ERK and PI3K-dependent manner, which underscores the interaction between cAMP, ERK, PI3K, and mTOR pathways. Second, forskolin application increases the fluorescence of a 5' TOP reporter in both the cell body and dendrites via mTOR, ERK and PI3K, indicating that cAMP activation is also coupled with an increased translation rate of 5' TOP containing mRNAs.

Induction of L-LTP in hippocampal slice cultures

To date, very few studies have examined late-phase LTP in organotypic hippocampal slices, even though this system is very useful for manipulation of protein expression. Indeed, organotypic slices can be maintained in culture for several days to weeks and therefore allow for the transfection and the long-term expression of fluorescent reporters (in this case, 5' TOP or mutated 5' TOP constructs). In previous reports (Muller *et al.* 1993; Leutgeb *et al.* 2003; Mellentin *et al.* 2006), LTP in slice culture has been studied mostly for a short period after LTP induction (approximately 1 h). At such time-point, changes observed at synapses are mostly associated to early-phase LTP rather than to the late-phase of LTP. In our study,

we were able to record fEPSP responses for up to 4 h, which corresponds to the maintenance phase of L-LTP, by using chemical induction by forskolin. Our results suggest that this chemical induction protocol in slice culture may be a useful tool for a detailed examination of molecular pathways implicated in L-LTP.

Interactions between cAMP, ERK, PI3K and mTOR pathways during L-LTP

It has previously been shown that cAMP and mTOR signaling pathways are both required for late-phase LTP in the hippocampus (Wong *et al.* 1999; Tang *et al.* 2002; Cammalleri *et al.* 2003; Wang *et al.* 2004) but it is still unclear whether these two pathways interact with each other. Several studies have shown that forskolin can induce a L-LTP that shares many features of electrically-induced L-LTP (Frey *et al.* 1993; Otmakhov *et al.* 2004). On the other hand, various papers demonstrate that rapamycin selectively blocks the late-phase LTP in the hippocampus (Tang *et al.* 2002; Cammalleri *et al.* 2003). Nevertheless, it has never been shown that mTOR blockade could also interfere with the late-phase of forskolin-induced LTP. Importantly, our results now demonstrate that forskolin-induced LTP is sensitive to rapamycin pre-treatment, suggesting that adenylate cyclase stimulation is activating the mTOR pathway and that this is a necessary step in forskolin-induced L-LTP. Consistent with cAMP activation acting through mTOR, we found that cAMP activation increased the phosphorylation of S6K and S6, two downstream effectors of mTOR, in a rapamycin-sensitive manner.

Various effectors could mediate interactions between cAMP and mTOR pathways. Previous studies have identified Akt/PKB and ERK as upstream regulators of mTOR (Gingras *et al.* 2001; Hay and Sonenberg 2004; Kelleher *et al.* 2004a; Lenz and Avruch 2005). Because in our study both phosphorylation of Akt and ERK were increased by forskolin treatment, it is likely that mTOR activation is downstream of these two known activators of mTOR (Fig. 7). While forskolin is not a natural stimulus, activation of adenylate cyclase is critical for ERK activation during both LTP and memory formation (Impey *et al.* 1998; Sindreu *et al.* 2007) and ERK is involved in activating the mTOR pathway during LTP (Kelleher *et al.* 2004a).

Using various biochemical approaches, several recent reports have clearly shown that there is an interaction between ERK and mTOR pathways in hippocampal slices during both LTP and long-term depression (LTD) (Kelleher *et al.* 2004a; Banko *et al.* 2006; Tsokas *et al.* 2007; Antion *et al.* 2008b). Indeed, phosphorylation of various effectors downstream of mTOR, including S6, S6K, and 4EBP1, are inhibited by treatment with PD98059 or UO126, two MEK/ERK inhibitors (Kelleher *et al.* 2004a; Banko *et al.* 2006; Antion *et al.* 2008b). Similarly, Tsokas *et al.* 2007 have demonstrated that HFS-induced phosphorylation of mTOR and translational proteins like eEF1A, S6, eEF2, and PABP1 is inhibited by incubation with PD98059 and UO126 (Tsokas *et al.* 2007). In our system, both phosphorylation of S6K at the mTOR site (Thr389) and S6 were prevented by addition of the specific MEK/ERK inhibitor PD98059, supporting the idea that forskolin activates the mTOR pathway in an ERK-dependent manner. Although our model depicts a direct interaction between ERK and mTOR pathways (Fig. 7), this might not be the case. Indeed, studies have suggested that activation of the mitogen-activated protein kinase (MAPK)/ERK pathway leads to phosphorylation of hamartin and tuberlin, two proteins forming the

tuberous sclerosis complex (TSC) complex and whose role is to inhibit signaling by mTOR (Roux *et al.* 2004). Other reports have also shown that the MAPK/ERK pathway might also act directly downstream of mTOR on proteins such as rpS6. Indeed, studies in S6K1^{-/-}/S6K2^{-/-} double knock-out (KO) cells have revealed that, in addition to S6K1 and S6K2, a MAPK-dependent kinase can also phosphorylate rpS6 at Ser235/Ser236 in an mTOR-independent mechanism (Pende *et al.* 2004). Subsequent work have identified the p90rsk as the kinases mediating the phosphorylation of rpS6 on these residues, and confirmed that Ras/ERK signaling promotes translation initiation (Roux *et al.* 2007).

Other studies have also suggested that cAMP activation during LTP is accompanied by the extracellular release of brain-derived neurotrophic factor (BDNF) (Patterson *et al.* 2001). Released BDNF could in turn activate TrkB receptors and the PI3K-Akt/PKB cascade (Patterson *et al.* 2001), and ultimately lead to the activation of the mTOR pathway (Takei *et al.* 2004). As phosphorylation of S6K at the mTOR site (Thr389) and S6 were also sensitive to LY294002, a specific PI3K inhibitor, our results not only suggest that S6K and S6 phosphorylation are downstream of ERK, but also of PI3K activation. Therefore, adenylate cyclase stimulation could trigger several cascades that lead to mTOR activation and translational activation (Fig. 7).

Although previous work has also suggested that ERK activation might be downstream of PI3K (Opazo *et al.* 2003; Tsokas *et al.* 2007), this might not be the case in our study. Indeed, we found that application of LY294002 did not affect the forskolin-induced increase in ERK phosphorylation. However, it is important to note that previous work used different LTP-inducing paradigms than cAMP stimulation (NMDA application, HFS-stimulation and TBS stimulation) and therefore, different cascades might be recruited. Alternatively, cross-talk between PI3K and ERK pathways may require stronger activation, like four trains of high-frequency stimulation (Tsokas *et al.* 2007).

L-LTP is associated with the activation of downstream targets of mTOR

Mammalian target of rapamycin effects are mediated by phosphorylation of both 4E-BPs and S6K, and by selective up-regulation of 5' TOP mRNAs. Previously, it has been shown that LTP- or LTD-inducing stimuli increase the phosphorylation of 4E-BPs, S6K, and S6, and increase the levels of TOP-encoded proteins (Cammalleri *et al.* 2003; Takei *et al.* 2004; Banko *et al.* 2005, 2006; Tsokas *et al.* 2005, 2007; Antion *et al.* 2008b); however, the relative role that these pathways play in LTP has not been determined. Further work has shown that genetic deletion of either 4EBP2, S6K1, or S6K2 lead to specific deficits in LTP and memory, thus confirming the importance of these downstream targets of mTOR (Banko *et al.* 2005; Antion *et al.* 2008a). Surprisingly, however, removal of S6K1 or S6K2 did not seem to have much impact on L-LTP, suggesting that other downstream targets of mTOR like 4EBP2 might be more crucial for the stabilization of L-LTP (Antion *et al.* 2008a). Here, we provide evidence that LTP may also specifically involve an increase in 5' TOP mRNAs through an mTOR-mediated pathway. It would be interesting in the future to determine which downstream pathway of TOR is required for mTOR-mediated synaptic plasticity.

On the other hand, as ERK, PI3K, and mTOR activation are also required for some forms of LTD (Gallagher *et al.* 2004; Hou and Klann 2004; Banko *et al.* 2006; Antion *et al.* 2008b), it

would be interesting to determine if different downstream pathways of TOR are important for distinct roles of mTOR-mediated translational regulation in synaptic plasticity. Recently, Sajikumar and Frey (2004) described the phenomenon of ‘cross-tagging’ in which proteins synthesized in response to L-LTP stimuli can be captured by synapses undergoing early-LTD or inversely. This raises the intriguing possibility that the same proteins may be synthesized in response to either L-LTP or late-LTD-inducing stimuli, and captured at synapses that have been appropriately tagged by synaptic activity.

Initially, it was reported that mTOR activation increased phosphorylation of S6K and S6 and that this was correlated with augmented translation rates of 5′ TOP containing mRNAs (Meyuhas 2000; Meyuhas and Hornstein 2000). However, recent evidence contests this model. Knockout mice lacking both S6K1 and S6K2 showed that up-regulation of 5′ TOP mRNAs in response to mitogens is maintained (Pende *et al.* 2004) and knock-in mutants of S6, lacking all S6 phosphorylation sites, also have normal mTOR-dependent regulation of 5′ TOP mRNAs (Ruvinsky *et al.* 2005). Thus, although S6K and S6 phosphorylation levels can still be used as readouts of the activity of the mTOR pathway, these data strongly suggest the existence of another as yet uncharacterized pathway linking mTOR activation and 5′ TOP mRNAs translation.

L-LTP is associated with an increase in 5′ TOP mRNAs translation

Recent reports have suggested that levels of some proteins that are encoded by 5′ TOP containing mRNAs are up-regulated during L-LTP and LTD (Tsokas *et al.* 2005, 2007; Poon *et al.* 2006; Liao *et al.* 2007; Antion *et al.* 2008b). However, although previous work has correlated LTP-inducing stimulation and increases in the translation of some 5′ TOP mRNAs [for example, eEF1α, see Tsokas *et al.* (2005, 2007)], no direct causal link has ever been reported. More precisely, it has not been shown that the augmentation observed in these studies were restricted to the 5′ TOP family. Indeed, it might be possible that LTP-inducing protocols cause a general increase in translation of all dendritically targeted mRNAs, rather than a selective up-regulation of 5′ TOP mRNAs. In this study, we thus specifically designed fluorescent reporters to investigate the regulation of 5′ TOP containing mRNAs during L-LTP by comparing two constructs that were identical except for a single residue change that determines TOP regulation (Avni *et al.* 1994). Interestingly, in the cell body of CA1 pyramidal cells, while levels of both reporters were increased by forskolin, only levels of the proteins encoded by the 5′ TOP-containing mRNAs were sensitive to rapamycin pre-treatment. Moreover, in the dendrites of CA1 pyramidal cells, only levels of the reporter encoded by the 5′ TOP-containing mRNAs were increased, again in an mTOR-dependent manner. Taken together, our results thus clearly suggest that 5′ TOP mRNAs are specifically regulated after LTP induction. A better understanding of the mechanism for TOP regulation downstream of mTOR will allow for selective blockade of this pathway and determining the importance of TOP regulation for LTP.

There are several possible explanations for the differential regulation of 5′ TOP mRNAs in dendrites versus cell bodies. First, different mechanisms for regulation of translation in the cell body and dendrites might be responsible for the more selective up-regulation of 5′ TOP mRNAs observed here. For example, the rate limiting steps for translation could be distinct,

and thus in dendrites, the process up-regulated by forskolin in a rapamycin-insensitive manner that acts on the non-TOP mRNAs is not present or not rate limiting. It should also be noted that the construct used for measuring dendritic regulation had the 3' UTR of MAP2, and this may add additional translational constraints. Thus, the rapamycin-insensitive process up-regulated by forskolin in the cell body may no longer be rate limiting for the construct encoding the 3' UTR of MAP2 as additional rate-limiting steps are now present. For example, translation of this construct in dendrites may now be regulated by removal of 3' UTR-mediated repression, and as this process may not be activated by forskolin, it could explain the loss of forskolin-mediated regulation of the non-TOP construct in dendrites. As the only difference between the TOP and non-TOP construct was the C–A conversion at the beginning of the TOP sequence, differences between the activation of these constructs by forskolin and their sensitivity to rapamycin can be directly attributed to regulation of the TOP sequence. Thus, in both cell bodies and dendrites, the TOP sequence provides an additional forskolin-induced rapamycin-sensitive regulation that is likely to be important for the physiological regulation of 5' TOP containing mRNAs.

We observed a differential sensitivity of the induction by forskolin to rapamycin between proximal and distal dendrites. A larger concentration of rapamycin was required to block the forskolin-induced increase in the 5' TOP reported construct at proximal dendrites. While it is possible that the higher concentration may affect some other pathways, rapamycin has been reported to be extremely specific for mTOR even at higher concentrations (Bain *et al.* 2007). It may be because of an increased concentration of mTOR at proximal sites. mTOR has been reported to be linked to gephyrin, a marker of inhibitory synapses that are enriched in proximal dendrites (Sabatini *et al.* 1999).

Up-regulation of the translational machinery during L-LTP

As described in the literature, the 5' TOP mRNAs family is mainly composed of ribosomal proteins and elongation factors and includes most components of the translational machinery (Meyuhas 2000; Meyuhas and Hornstein 2000). Ribosome biogenesis is highly energy costing and is therefore finely regulated to meet the changing requirements for the translational machinery. It has been shown that the presence of the TOP motif in the 5' untranslated region allows for the rapid up-regulation of this class of mRNAs during situations that require high rates of protein synthesis, such as cell proliferation or differentiation (Meyuhas and Hornstein 2000). The shift of 5' TOP mRNAs from the subpolysomal fraction to the polyribosomal pool can be induced by over-expression of a TOP message, suggesting that 5' TOP mRNAs translation is regulated by an as yet unidentified repressor (Biberman and Meyuhas 1999). Although modulation of 5' TOP mRNAs synthesis has been extensively associated with cell growth and related processes, recent work has suggested that this pathway might also be important for the stabilization of LTP in vertebrates (Tsokas *et al.* 2005, 2007; Poon *et al.* 2006; Liao *et al.* 2007) and long term facilitation in *Aplysia* (Khan *et al.* 2001; Moccia *et al.* 2003). Indeed 5' TOP containing mRNAs are a major class of mRNAs sent out in processes in both vertebrates (Poon *et al.* 2006) and invertebrates (Moccia *et al.* 2003; Moroz *et al.* 2006). Moreover, ribosomal proteins were a major class of proteins translationally up-regulated in

synaptoneurosome after BDNF treatment, and this effect was dependent on activation of mTOR (Liao *et al.* 2007).

In *Aplysia*, rapamycin-sensitive local protein synthesis is important for the stabilization of long-term changes at synapses (Casadio *et al.* 1999). While complete ribosome biogenesis presumably cannot occur in dendrites, it is possible that during transport, ribosomes lose peripheral proteins, and that local translation of these subunits at stimulated synapses is required to activate dormant ribosomes (Moccia *et al.* 2003). Increases in the number of translationally competent ribosomes via the selective up-regulation of 5' TOP mRNAs synthesis would then serve to specifically boost the translational capacity of activated synapses for a prolonged period and thus mark the synapse for persistence of changes. A meta-activation of local translation may be important to promote the translation of newly delivered mRNAs, and thus reconcile the requirement for local translation with the requirement for transcription and transport of newly synthesized mRNAs to synapses.

Taken together, our results demonstrate for the first time that 5' TOP mRNAs are differentially regulated after LTP-inducing stimulation in an mTOR-dependent manner. Although we cannot rule out the implication of other mTOR targets, our present data clearly suggest that up-regulation of the translational machinery via the selective synthesis of 5' TOP mRNAs, might play an important role for the stabilization of L-LTP.

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Abbreviations used

5' TOP	5' terminal oligopyrimidine tract
ACSF	artificial cerebrospinal fluid
BDNF	brain-derived neurotrophic factor
ERK	extracellular signal-regulated kinase
fESPS	field excitatory post-synaptic potentials
HEK	human embryonic kidney
LTD	long-term depression
L-LTP	late-LTP
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase

mTOR	mammalian target of rapamycin
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
UTR	untranslated region

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Fig. 1.

Forskolin (Fsk) induces late-long-term potentiation (LTP) in organotypic hippocampal slices. (a) Time plot of field excitatory post-synaptic potential (fEPSP) responses for group data with representative recordings above, showing that four trains of high-frequency stimulation elicit LTP of fEPSP responses that persists for at least 4 hours (grey circles, $n = 7$) in acute slices. In the presence of 200 nM rapamycin (RAP, black bar), an inhibitor of the mammalian target of rapamycin (mTOR), the late phase of LTP is specifically inhibited (black circles, $n = 6$). (b) Summary bar graph indicating that LTP in rapamycin (Rap)-treated slices (black bars) is significantly reduced 180–240 min post-tetanus as compared to vehicle-treated slices (grey bars; $p < 0.05$). (c) Time plot of fEPSP responses showing that the application of 50 μ M Fsk (grey bar) produces a robust and sustained LTP (grey circles, $n = 6$) in cultured slices. Fsk-induced LTP was prevented by the application of 200 nM Rap (black circles, $n = 7$). (d) Summary bar graph indicating that LTP is significantly reduced 120–240 min post-Fsk ($p < 0.01$) in Rap-treated slices (black bars) as compared to vehicle-treated slices (grey bars). * $p < 0.05$; ** $p < 0.01$; NS, not significant.

Fig. 2.

Forskolin (Fsk) increases the activity of several components of the mammalian target of rapamycin (mTOR) pathway in hippocampal slices. (a) Western blot analysis of hippocampal extracts reveals that S6K phosphorylation (Thr389) is significantly increased by the application of 50 μ M Fsk ($p < 0.01$). This increase is completely blocked by pre-treatment of hippocampal slices with 200 nM rapamycin (Fsk + Rap). (b) Fsk also increases the phosphorylation levels of the ribosomal protein S6 (Ser235/236), a TOP-mRNA encoded protein ($p < 0.05$) which was blocked by Rap. (c) Akt phosphorylation is significantly increased by 50 μ M Fsk ($p < 0.001$) but this augmentation is not prevented by the addition of 200 nM Rap. (d) Similarly, phosphorylation of extracellular signal-regulated kinase (ERK) (Tyr204) is increased by treatment with Fsk ($p < 0.001$) but this effect was not blocked by pre-treatment with Rap ($p < 0.001$) indicating that Rap only affected phosphorylation of proteins downstream of mTOR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, non-significant.

Fig. 3.

Forskolin selectively activates a 5' terminal oligopyrimidine (5' TOP) reporter via mammalian target of rapamycin (mTOR) in human embryonic kidney (HEK) cells. HEK cells transfected with fluorescent 5' TOP and mutated 5' TOP reporters were tested for serum-induced translation. Summary bar graph showing that after an overnight starving, serum addition selectively increases the fluorescence of a 5' TOP reporter ($p < 0.05$) and this is blocked by 20 nM rapamycin (right bars). Mutated 5' TOP reporter is not stimulated by serum (left bars). Addition of rapamycin reveals the existence of a rapamycin-insensitive increase in the level of the mutated 5' TOP reporter ($p < 0.05$). * $p < 0.05$; NS, not significant.

Fig. 4.

Forskolin (Fsk) increases somatic 5' terminal oligopyrimidine (5' TOP) mRNAs translation via mammalian target of rapamycin (mTOR) in slice cultures. (a and b) Confocal images (a) and summary bar graph (b) from CA1 pyramidal cells transfected with the 5' TOP reporter. The fluorescence levels are significantly increased in cells from slices treated with 50 μ M Fsk, as compared to cells from vehicle-treated slices ($p < 0.01$). This increase in fluorescence is prevented by pre-treatment with 200 nM rapamycin (Rap). (c and d) Similar experiments showing that the fluorescence levels of a mutated 5' TOP reporter are significantly increased in cells from slices treated with Fsk ($p < 0.05$), but, in contrast, this augmentation is not blocked by the pre-treatment with Rap. Scale bar: 20 μ m. * $p < 0.05$; ** $p < 0.01$; NS, not significant.

Fig. 5.

Forskolin (Fsk) increases dendritic 5' terminal oligopyrimidine (5' TOP) mRNAs translation via mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase and extracellular signal-regulated kinase in slice cultures. (a and b) Confocal images (a) and summary plot (b) showing that the fluorescence levels of a 5'-TOPMAP2- 3' UTR reporter are significantly increased in dendrites of pyramidal cells by the application of 50 μM Fsk. This augmentation is prevented by pre-treatment with 200 nM rapamycin. (c) Summary bar graph illustrating that Fsk-induced increase is significant from proximal (at 10 μm, $p < 0.001$) to distal regions (at 100 μm, $p < 0.05$). (d and e) Similar experiments indicating that, in contrast, fluorescence levels of a mutated 5'-TOP-MAP2-3' UTR reporter are not increased by Fsk with or without rapamycin (Rap) pre-treatment. (f) Summary bar graph showing that there is no significant difference in fluorescence throughout the dendritic length in all conditions. (g and h) Confocal images (g) and summary plot (h) from separate experiments showing that the fluorescence levels of a 5'-TOP-MAP2-3' UTR reporter are significantly increased in dendrites of pyramidal cells by the application of 50 μM Fsk and that this augmentation is prevented by pre-treatment with 50 μM PD98059, 50 μM LY294002, or a high dose of Rap (1 μM). (i) Summary bar graph illustrating that Fsk-induced increase is significant from proximal (at 10 μm, $p < 0.001$) to distal regions (at 100 μm, $p < 0.05$). Scale bar: 10 μm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant.

Fig. 6.

Forskolin (Fsk) activates the mammalian target of rapamycin (mTOR) pathway in phosphatidylinositol 3-kinase- and extracellular signal-regulated kinases (ERK)-dependent manner in slice cultures. (a) Western blot analysis of hippocampal extracts reveals that S6K phosphorylation at the mTOR-dependent site (Thr389) is significantly increased by the application of 50 μ M Fsk ($p < 0.05$). This increase is completely blocked by pre-treatment of hippocampal slices with 50 μ M PD98059 (Fsk + PD), 50 μ M LY294002 (Fsk + LY), and a high dose (1 μ M) of rapamycin (Fsk + Rap). (b) Fsk also significantly increases ($p < 0.05$) S6K phosphorylation at the MEK/ERK-dependent site (Thr421/Ser424). In contrast, this increase is blocked by the addition of PD98059, but not by LY294002 ($p < 0.05$) or Rap ($p < 0.05$). (c) Similarly, phosphorylation of ERK (Tyr204) is increased by treatment with Fsk ($p < 0.05$) and this effect was only blocked by pretreatment with PD98059 and not by LY294002 ($p < 0.05$) or Rap ($p < 0.05$). (d) Fsk also significantly augments ($p < 0.01$) the phosphorylation levels of the ribosomal protein S6 (Ser235/236), an effect that is prevented by the addition of PD98059, LY294002, or Rap. * $p < 0.05$; ** $p < 0.01$.

Fig. 7.

A model for the up-regulation of 5' terminal oligopyrimidine (5' TOP) mRNAs translation during late-long-term potentiation (LTP). During LTP-inducing stimulation, NMDA receptors are recruited by high-frequency synaptic activity and allow the entry of calcium into the post-synaptic neuron. This in turn activates adenylate cyclase (AC) and leads to the production of cAMP and the activation of cAMP-dependent protein kinase (PKA). PKA can then activate the mammalian target of rapamycin (mTOR). The precise mechanism involved remains unclear but PKA could activate mTOR via at least two pathways. First, PKA could activate the extracellular signal-regulated kinase (ERK) pathway. ERK has previously been shown to be required for the activation of downstream targets of mTOR such as 4E-BP and S6 (Kelleher *et al.* 2004a). Alternatively, cAMP production could cause the release of brain-derived neurotrophic factor (BDNF). Released BDNF can in turn activate TrkB receptors and the phosphatidylinositol 3-kinase (PI3K)/Akt-PKB pathway, which has also been shown to activate mTOR (Patterson *et al.* 2001; Takei *et al.* 2004). Finally, activation of mTOR pathway leads to the selective up-regulation of 5' TOP mRNAs. Our results suggest that a selective increase in 5' TOP mRNAs translation may be crucial for late-LTP stabilization. CaM, calmodulin; CREB, cAMP responsive element binding protein; CRE, cAMP responsive element.