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Tumstatin, an Endothelial Cell-Specific Inhibitor of Protein Synthesis

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Tumstatin is a 28-kilodalton fragment of type IV collagen that displays both anti-angiogenic and proapoptotic activity. Here we show that tumstatin functions as an endothelial cell-specific inhibitor of protein synthesis. Through a requisite interaction with α V β 3 integrin, tumstatin inhibits activation of focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3-kinase), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR), and it prevents the dissociation of eukaryotic initiation factor 4E protein (eIF4E) from 4E-binding protein 1. These results establish a role for integrins in mediating cell-specific inhibition of cap-dependent protein synthesis and suggest a potential mechanism for tumstatin's selective effects on endothelial cells.

Apoptosis, or programmed cell death, is regulated in part at the level of protein synthesis (1–4). We have been studying a basement membrane-derived angiogenesis inhibitor called tumstatin, which selectively stimulates apoptosis of endothelial cells (5–8). Tumstatin is a NC1 domain fragment

of the type IV collagen α 3 chain. It binds to α V β 3 integrin and has been shown to inhibit the growth of tumors in mouse models (5–8).

Because apoptosis is generally associated with inhibition of cap-dependent translation, we investigated the effect of tumsta-

tin on protein synthesis in endothelial cells (9). We used full-length tumstatin and three recently identified active subfragments (Tum-5, T3, and T7; as determined by using *in vivo* angiogenesis assays) in these experiments (10). At maximal dose (22.7 μ M), tumstatin peptide T3 was found to inhibit protein synthesis in cultured bovine endothelial cells by 45% (Fig. 1A). At molar equivalent concentrations (4.5 μ M), all tumstatin peptides inhibited protein synthesis by 25 to 30% (Fig. 1, B and C) (11). Rapamycin, a well-characterized mTOR/protein synthesis inhibitor (12), inhibited

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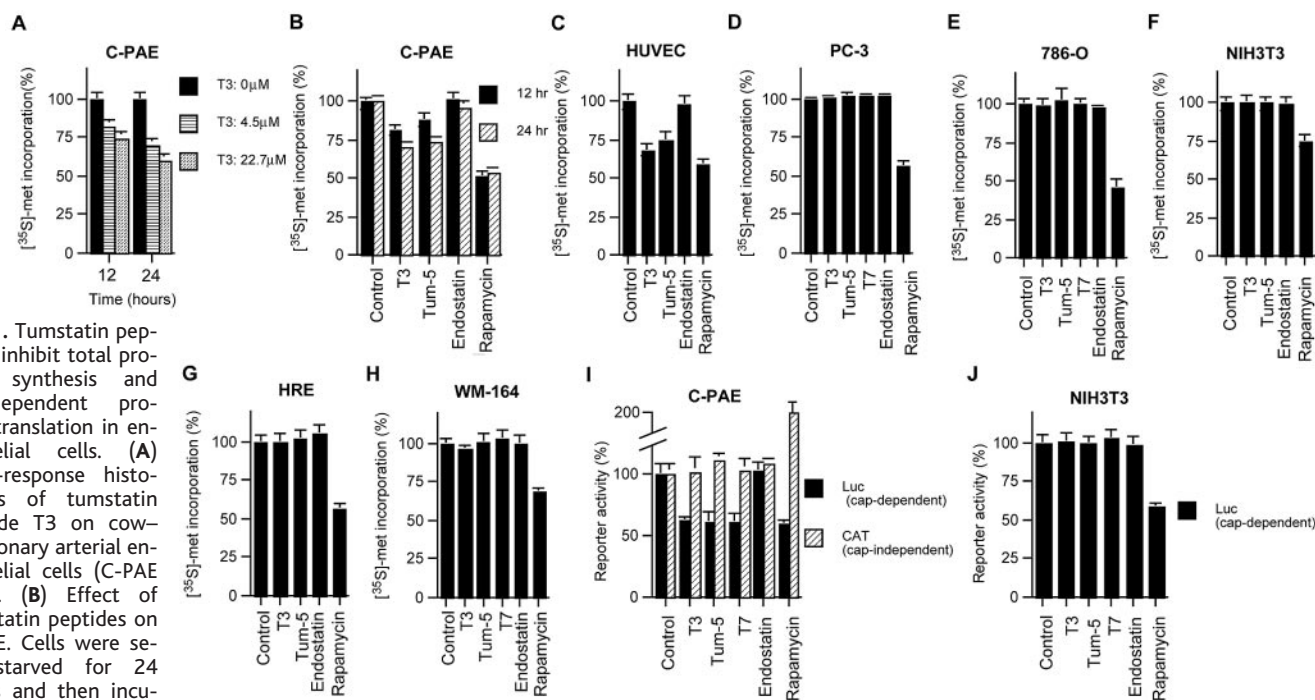


Fig. 1. Tumstatin peptides inhibit total protein synthesis and cap-dependent protein translation in endothelial cells. (A) Dose-response histograms of tumstatin peptide T3 on cow-pulmonary arterial endothelial cells (C-PAE cells). (B) Effect of tumstatin peptides on C-PAE. Cells were serum-starved for 24 hours and then incubated with medium containing 10% fetal calf serum (FCS) for 24 hours in the presence of T3 peptide (4.5 μ M), tum-5 (4.5 μ M), endostatin (4.5 μ M), or rapamycin (100 ng/ml). Total protein synthesis was measured as in (A). (C to H) Effect of tumstatin peptides on human umbilical vein endothelial cells (HUVEC) and nonendothelial cells. (I) Effect of tumstatin peptides on cap-dependent translation in C-PAE cells. C-PAE cells were serum-starved for 24 hours and then transiently transfected with 1.5 μ g of pcDNA3-LUC-pol-CAT. After 3 hours, medium was replaced with culture medium containing 10% FCS in

the presence of T3 peptide (4.5 μ M), tum-5 (4.5 μ M), T7 peptide (4.5 μ M), endostatin (4.5 μ M), or rapamycin (100 ng/ml). After 21 hours, total cell extracts were prepared and assayed for both luciferase and CAT activity. Reporter activity is expressed as percent relative to the control group. (J) NIH 3T3 cells were used to examine cap-dependent translation as in (I). These experiments were repeated three times, and representative data are shown. Each column consists of mean \pm SEM of triplicates. Additional experimental details are provided in (11).

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protein synthesis by 48% in these experiments, whereas endostatin, another matrix-derived angiogenesis inhibitor (13–15), had no effect on protein synthesis (Fig. 1, B and C). Tumstatin peptides did not inhibit protein synthesis in nonendothelial cells such as PC-3 prostate carcinoma cells, 786-O renal carcinoma cells, NIH 3T3 fibroblasts, primary human renal epithelial cells (HRE), or WM-164 human melanoma cells (Fig. 1, D to H). In contrast, rapamycin inhibited protein synthesis in all cells tested (Fig. 1, B to H).

To investigate whether tumstatin-mediated inhibition of protein synthesis was cap-dependent (dependent on mRNA 5' cap structure, M⁷GpppX), we transfected endothelial cells with a plasmid encoding dicistronic reporter mRNA under the control of the cytomegalovirus promoter. The reporter mRNA consisted of luciferase (LUC) mRNA and chloamphenicol acetyltransferase (CAT) mRNA separated by an internal ribosomal entry site (IRES) derived from the untranslated region of poliovirus (11, 12, 16). Expression of this plasmid (pcDNA3-LUC/pol/CAT) resulted in cap-dependent translation of LUC mRNA and cap-independent translation of CAT mRNA (12, 16). Tumstatin peptides decreased cap-dependent translation of LUC by 38% in endothelial cells, comparable to the effect of rapamycin, and endostatin had no effect (Fig. 1I). Again, this effect was specific to endothelial cells (Fig. 1J). Importantly, cap-independent translation (CAT activity) was not altered by tumstatin peptides (Fig. 1I). Consistent with previous reports suggesting that rapamycin stimulates the trans-

lation of mRNAs containing IRES (4, 17, 18), rapamycin induced cap-independent translation in endothelial cells (Fig. 1I). Tumstatin did not alter mRNA levels in the endothelial cells (19).

Tumstatin-induced apoptosis of endothelial cells requires its binding to α V β 3 integrin (6–8). To determine if α V β 3 integrin was also involved in the inhibition of cap-dependent translation by tumstatin, we isolated endothelial cells from lungs of β 3 integrin-deficient mice and their wild-type counterparts (11, 20, 21). Tumstatin peptides inhibited cap-dependent protein synthesis in wild-type cells (β 3^{+/+}) but not in β 3 integrin-deficient (β 3^{-/-}) cells, whereas rapamycin's activity was independent of β 3 expression status (Fig. 2, C and D and G and H). Tumstatin peptides did not inhibit protein synthesis in mouse embryonic fibroblasts expressing α V β 3 integrin (Fig. 2, E and F) (20), indicating that α V β 3 integrin expression is essential but not sufficient for the tumstatin activity. Further studies are required to identify the α V β 3 integrin-associated factors that determine tumstatin's endothelial cell specificity.

We next performed experiments to elucidate the role of tumstatin in signaling pathways involved in the inhibition of protein synthesis. In many cell types, including endothelial cells, ligand binding to integrin induces phosphorylation of focal adhesion kinase (FAK), leading to the activation of various signaling molecules (22, 23). Phosphorylated FAK interacts with and activates phosphatidylinositol 3-kinase (PI3-kinase) and protein kinase B (PKB/Akt; downstream of PI3-kinase), leading to cell

survival (22, 24). Inhibition of PI3-kinase in endothelial cells has been shown to repress protein synthesis (25).

Tumstatin peptides inhibited phosphorylation of FAK induced in endothelial cells by attachment to vitronectin (Fig. 3A) (11). Activation of PI3-kinase and Akt was also inhibited by treatment with tumstatin peptides (Fig. 3, B and C) (11). Rapamycin/FKBP-target 1 protein (RAFT1), also known as mammalian target of rapamycin (mTOR) and activated by Akt, directly phosphorylates eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) (26, 27). Unphosphorylated 4E-BP1 interacts with eIF4E and inhibits cap-dependent translation (28). Stimulation of cells with growth factors or serum induces phosphorylation of 4E-BP1, resulting in its dissociation from eIF4E to relieve translational inhibition (27, 28). We found that tumstatin peptides suppressed mTOR kinase activity and thus inhibited phosphorylation of 4E-BP1 (Fig. 3D) (11). Inhibition of 4E-BP1 phosphorylation enhanced 4E-BP1 binding to eIF4E (Fig. 3E) (11), leading to inhibition of cap-dependent translation. By contrast, in WM-164 melanoma cells expressing α V β 3 integrin, inhibition of FAK, Akt, and mTOR was not observed (Fig. 3, A and C to E). We also investigated the potential role of the mitogen-activated protein kinase pathway in tumstatin's effects on protein synthesis. Phosphorylation of extracellular regulated kinase (ERK)1/2 upon vitronectin attachment or stimulation with VEGF (vascular endothelial growth factor) was not altered by tumstatin peptides in C-PAE cells (19).

To confirm the importance of the mTOR pathway in tumstatin's effect on protein

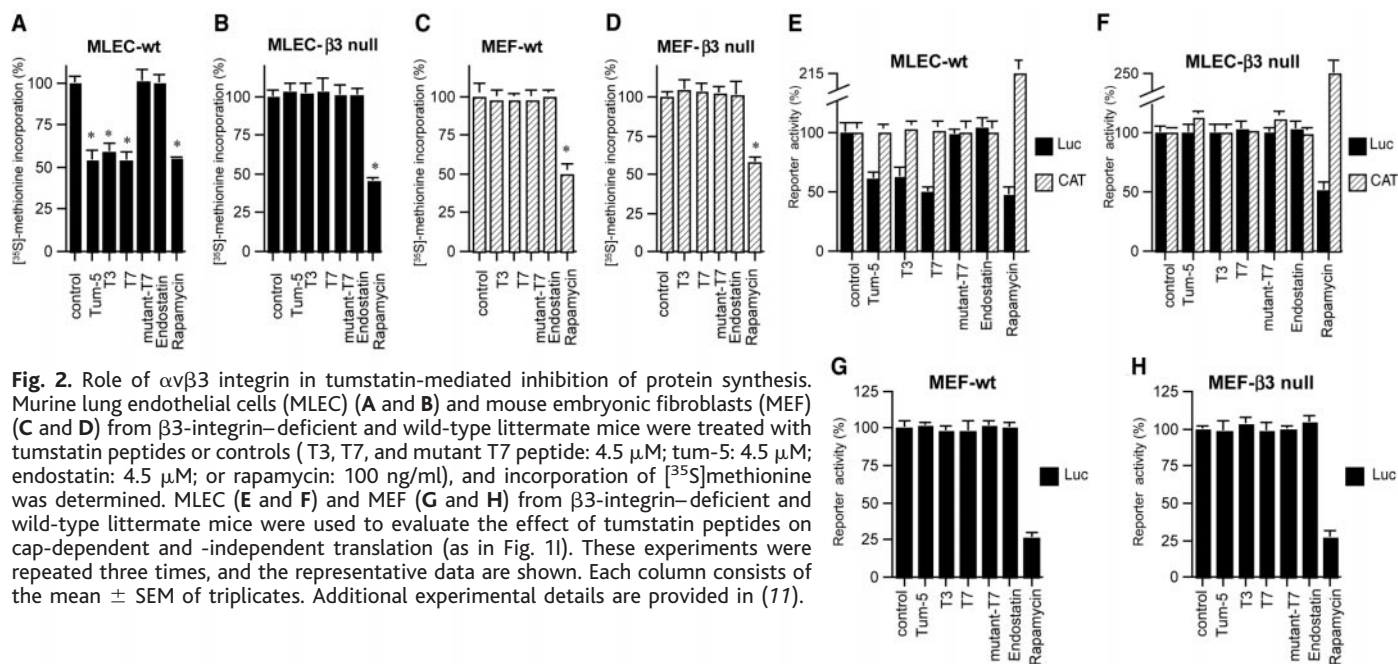
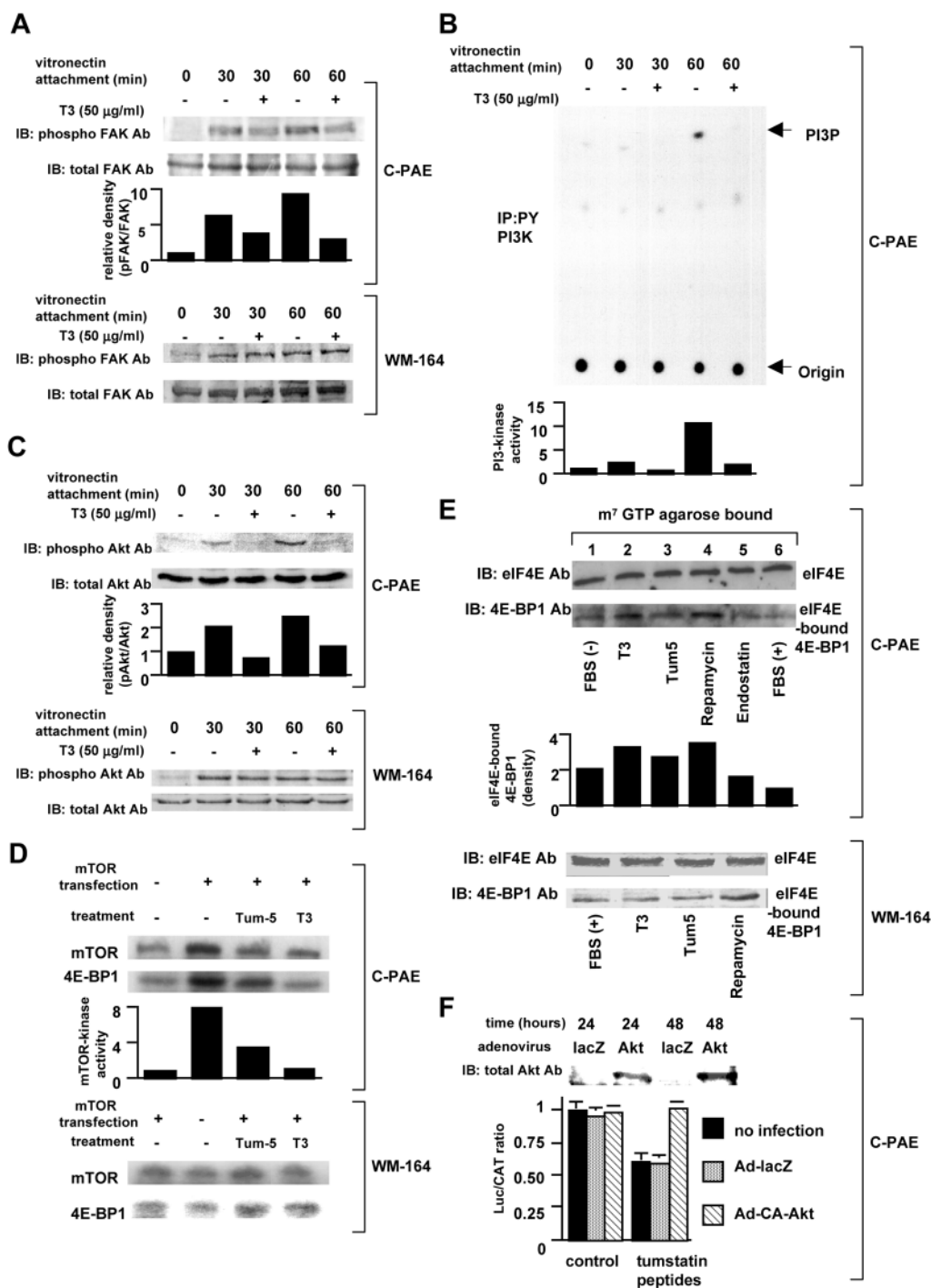


Fig. 2. Role of α V β 3 integrin in tumstatin-mediated inhibition of protein synthesis. Murine lung endothelial cells (MLEC) (A and B) and mouse embryonic fibroblasts (MEF) (C and D) from β 3-integrin-deficient and wild-type littermate mice were treated with tumstatin peptides or controls (T3, T7, and mutant T7 peptide: 4.5 μ M; tum-5: 4.5 μ M; endostatin: 4.5 μ M; or rapamycin: 100 ng/ml), and incorporation of [³⁵S]methionine was determined. MLEC (E and F) and MEF (G and H) from β 3-integrin-deficient and wild-type littermate mice were used to evaluate the effect of tumstatin peptides on cap-dependent and -independent translation (as in Fig. 1I). These experiments were repeated three times, and the representative data are shown. Each column consists of the mean \pm SEM of triplicates. Additional experimental details are provided in (17).

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Fig. 3. Tumstatin peptides down-regulate PI3-kinase, Akt, and mTOR signaling pathway, leading to decreased phosphorylation of 4E-BP1. **(A)** Cow pulmonary artery endothelial cells (C-PAEs) or WM-164 melanoma cells were serum-starved for 30 hours and trypsinized. Cells in suspension were preincubated with T3 peptide (50 $\mu\text{g/ml}$) for 15 min, and then allowed to attach onto vitronectin-precoated dishes in serum-free conditions for 30 to 60 min. Total cell extracts were prepared, and SDS-polyacrylamide gel electrophoresis and Western blotting with antibodies to FAK (anti-FAK) and anti-phosphorylated FAK was performed. **(B)** C-PAEs were treated with T3 peptide and allowed to attach on vitronectin-coated dishes. Cell lysates were immunoprecipitated (IP) with 4G10 and subjected to PI3-kinase assay (PI3K). **(C)** Western blotting with anti-Akt and anti-phosphorylated Akt was performed as in (A) with C-PAE and WM-164 cells. **(D)** C-PAEs were transiently transfected with hemagglutinin (HA)-mTOR and treated with T3 peptide or Tum-5. Cell lysates were immunoprecipitated with anti-HA and incubated with glutathione-S-transferase (GST)-4E-BP1. The kinase reaction was performed in the presence of [γ - ^{32}P]ATP. Reactions were resolved by SDS-PAGE and analyzed by autoradiography. The kinase activity of mTOR in autophosphorylation (upper panel) and phosphorylation of 4E-BP1 (lower panel) are shown. The kinase activity of mTOR was also examined by using WM-164 cells. **(E)** C-PAEs or WM-164 cells were treated with T3 peptide, Tum-5, rapamycin, or endostatin, and cell lysates were incubated with $m^7\text{GTP}$ -agarose beads. Samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-eIF4E (upper panel) and anti-4E-BP1 (lower panel). **(F)** C-PAEs were infected with adenoviral vectors encoding lactose Z operon (lacZ) or a mutant Akt gene that produces a constitutively active protein. After 24 to 48 hours, cells were harvested and the level of Akt was determined by Western blotting (upper panel). After infection of cells for 24 hours, C-PAEs were serum-starved, transfected with pcDNA-LUC-pol-CAT, and treated with tumstatin peptides in the presence of medium containing 10% FCS, and then cap-dependent translation was determined. The luciferase



activity relative to CAT activity is shown. These experiments were repeated two to three times, and the representative data (mean \pm SEM) are shown. Additional experimental details are provided in (11).

synthesis, we introduced constitutively active Akt into endothelial cells using recombinant adenoviruses (11). Inhibition of cap-dependent translation by tumstatin peptides was overcome by overexpression of constitutively active Akt (Fig. 3F). These data are consistent with the hypothesis that the tumstatin peptides inhibit endothelial protein synthesis through negative regulation of

mTOR signaling. An alternative hypothesis is that the tumstatin/ $\alpha\text{v}\beta 3$ integrin-induced negative signals counteract growth factor-initiated cell survival signals through cross talk between these two pathways.

Currently there are more than 20 angiogenesis inhibitors in clinical trials for the treatment of cancer. These inhibitors fall into two general categories: (i) small molecules or antibodies

that target a pro-angiogenic product of a tumor cell (e.g., VEGF) or (ii) endogenous proteins in the blood (e.g., thrombospondin-1, angiostatin, interferon- β) or in the tissues (e.g., endostatin, tumstatin) that target vascular endothelial cells. The endogenous inhibitors have shown high selectivity for inhibition of proliferating endothelial cells in the tumor bed in both animal and human studies. However, the molecular mech-

anisms underlying this specificity have not been clear. Our findings indicate that tumstatin is a potent angiogenesis inhibitor because it specifically inhibits protein synthesis in vascular endothelial cells in a $\alpha V\beta 3$ integrin-dependent manner, leading to endothelial cell-specific apoptosis.

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- as recombinant tum-5 in *Escherichia coli* (7). Human endostatin was produced in yeast (15). We used only soluble protein with a low endotoxin level (less than 50 endotoxin units/mg). T3 peptide (LQRFTMPFLFCNVNDVCNF), T7 peptide (TMPFLFCNVNDVCNFASRNDYSYWL) consisting of residues 69 to 88 and 74 to 98 of tumstatin, respectively, and mutant T7 peptide (TMPFMFCNINNVCFASRNDYSYWL) were synthesized as in (6, 8).
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Mediation of Hippocampal Mossy Fiber Long-Term Potentiation by Presynaptic I_h Channels

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Hippocampal mossy fiber long-term potentiation (LTP) is expressed presynaptically, but the exact mechanisms remain unknown. Here, we demonstrate the involvement of the hyperpolarization-activated cation channel (I_h) in the expression of mossy fiber LTP. Established LTP was blocked and reversed by I_h channel antagonists. Whole-cell recording from granule cells revealed that repetitive stimulation causes a calcium- and I_h -dependent long-lasting depolarization mediated by protein kinase A. Depolarization at the terminals would be expected to enhance transmitter release, whereas somatic depolarization would enhance the responsiveness of granule cells to afferent input. Thus, I_h channels play an important role in the long-lasting control of transmitter release and neuronal excitability.

A remarkable property of many excitatory synapses in the central nervous system is their ability to undergo activity-dependent, long-lasting increases in synaptic strength, referred to as LTP, a process that may underlie certain forms of memory. In most cases LTP requires the activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors (1, 2). However, LTP at hippocampal mossy fiber synapses is independent of NMDA receptor activation (3, 4) and is proposed to be initiated by a rise in presynaptic calcium (4–6) [but see (7)], which results in a per-

sistent increase in the probability of transmitter release (8, 9). The mechanism involved in the persistent increase in evoked release is not clear, but considerable evidence supports a role for adenosine 3',5'-monophosphate (cAMP) (10–13). In addition, genetic deletion of the synaptic vesicle-associated proteins Rab3A or RIM (which binds to Rab3A) also abolishes mossy fiber LTP (14, 15). Recent results have shown that the presynaptic facilitation of synaptic transmission at the crustacean neuromuscular junction by cAMP involves I_h channels (16). Because cAMP is proposed to play a role in mossy fiber LTP, we examined a possible role for I_h in this form of plasticity.

Throughout this study we used the specific I_h channel blocker ZD7288 to examine the role of I_h (17). We compared the magnitude

of mossy fiber LTP in control slices to that obtained in slices incubated in 1 μ M ZD7288 (18). Tetanization of the mossy fibers in ZD7288 caused a transient posttetanic potentiation that decayed over a 10-min period toward control values ($110 \pm 11\%$, 25 to 30 min, $n = 6$). In contrast, in control slices tetanization caused a $192 \pm 7\%$ ($n = 9$) potentiation (Fig. 1A). Elevating cAMP causes a large enhancement in mossy fiber synaptic transmission that occludes LTP (10, 11). In the present experiments, the adenylyl cyclase activator forskolin (50 μ M, 5 min) caused a large enhancement in control conditions ($275 \pm 36\%$, 25 to 30 min, $n = 14$). This enhancement was strongly reduced by prior incubation of the slices in ZD7288 ($135 \pm 9\%$, $n = 6$) (Fig. 1B).

Blockade of both LTP and the cAMP-mediated enhancement by ZD7288 suggests that I_h channels are involved downstream of both tetanus and cAMP elevation and could perhaps be important for the maintenance of synaptic enhancement. ZD7288 was thus applied after the tetanus or application of forskolin. Because of the slow onset of action of 1 μ M ZD7288, we increased the concentration to 50 μ M (19). We performed three types of interleaved experiments: ZD7288 alone, LTP alone, and ZD7288 applied after the induction of LTP. These three sets of experiments are superimposed in Fig. 2A. ZD7288 reversed LTP over a period of 20 to 30 min. The decrease in paired pulse facilitation associated with LTP was also reversed (control, $81 \pm 4\%$; ZD7288, $104 \pm 6\%$). To confirm that ZD7288 reverses established LTP, we made recordings from two independent mossy fiber inputs so that we could simultaneously record the effects of ZD7288 on baseline responses and LTP (Fig. 2B). Again, ZD7288 completely reversed LTP. The fors-

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