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Effect of Calpain Inhibitors on Ca^{2+}-Induced Suppression of Neurite Outgrowth in Isolated Hippocampal Pyramidal Neurons

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ABSTRACT

Ca^{2+} is an important regulator of neurite elongation and growth cone movements but the mechanism (s) mediating these Ca^{2+}-dependent effects is unclear. Since cytoskeletal proteins are rapidly degraded by Ca^{2+}-dependent proteinases (calpains) *in vitro* and *in vivo*, we investigated whether Ca^{2+}-induced pruning or regression of neuronal processes is mediated by calpains. Isolated hippocampal pyramidal-like neurons were cultured and the ability of the membrane-permeable calpain inhibitors EST (etyl (+)-(2S,3S)-3-[(S)-methyl-1-(3-methlbutylcarbamoyl)-butylcarbamoyl]2-oxiranecarboxylate) and MDL 28170 (carbobenzoxyl-Val-Phe-H) to block the Ca^{2+} ionophore A23187-induced suppression in neurite outgrowth was investigated. Addition of 100 nM A23187 to the culture medium resulted in a retraction of dendrites without altering axonal elongation. The addition of 300 nM A23187 to the culture medium resulted in a signiciant decrease in the rate of axonal elongation as well as a retraction of dendritic processes. Administration of EST (5 or 20 μM) to the culture medium completely blocked the pruning effect of 100 nM A23187 on dendrites and of 300 nM A23187 on axons, while EST alone did not significantly affect neurite outgrowth rate. MDL 28170 (20 μM) showed the same effect as EST in preventing ionophore-induced pruning of dendrites and axons at 100 nM and 300 nM concentrations, respectively, of A23187. EST (20 μM) did not block the A23187-induced rise of $[Ca^{2+}]_i$ as measured with fura-2. These results show that Ca^{2+}-induced pruning of neurites in isolated hippocampal pyramidal neurons is mediated by calpains.

Key Words: Calpain, Neurite outgrowth, Calcium, Growth cone

INTRODUCTION

A variety stimuli can modulate growth cone morphology and subsequent neurite outgrowth. Neuronal growth can be influenced by neurotransmitters (Lipton and Kater, 1989), electrical activity (Cohan and Kater, 1986; Fields *et al.*, 1990), activity of target tissues (Vrbova and Lowrie, 1989), growth substrate (Hynes and Lander, 1992), and growth factors (Mattson, 1990). Intracellular Ca^{2+} can act as a key nodal point for the integration of multiple, simultaneously presented environmental stimuli. Certain neurotransmitters and membrane depolarization both cause an influx of Ca^{2+} that suppresses neurite outgrowth (Lipton and Kater, 1989; Cohan and Kater, 1986; Connor *et al.*, 1988; Mattson *et al.*, 1988b), which is ultimately based on cytoskeletal motility, stability, and architecture (Lankford and Letourneau, 1989).

The mechanism(s) mediating these Ca^{2+}-dependent changes in the neuronal cytoskeleton is as yet undefined. It has been proposed that Ca^{2+} af-

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fects the cytoskeleton by altering F-actin stability directly (Lankford and Letourneau, 1989) or by activating calmodulin-dependent enzymes (Polak *et al.*, 1991). In neuronal and non-neuronal systems, increased intracellular Ca^{2+} activates calpains *in vitro* (Mehdi *et al.*, 1988; Di Stasi *et al.*, 1991) and *in vivo* (Siman *et al.*, 1989). These Ca^{2+}-dependent proteinases have been isolated from a variety of vertebrate tissues and are present in high levels in brain and other nervous tissues (see Mellgren and Murachi, 1990; Croall and DeMartino, 1991 for reviews). Two isozymes, calpain I and calpain II, which differ in sensitivity to Ca^{2+}, are present, as well as an endogenous inhibitor, calpastatin. The calpains preferentially degrade cytoskeletal proteins, such as neurofilament proteins (Nixon *et al.*, 1986), brain spectrin (Siman *et al.*, 1989; Di Stasi *et al.*, 1991), actin-binding proteins (Nixon *et al.*, 1986; Fox *et al.*, 1991), and tubulin and microtubule-associated proteins (Billger *et al.*, 1988; Litersky and Johnson, 1992). They are distributed throughout all regions of neurons (Perlmutter *et al.*, 1990; Fukuda *et al.*, 1990) and are associated with the cytoskeleton in cell processes of hippocampal neurons (Perlmutter *et al.*, 1988). Reduction of calpain activity is correlated with stimulation of neurite outgrowth in PC12 (Oshima *et al.*, 1989) and neuroblastoma (Shea *et al.*, 1991, 1992) cells in culture. Furthermore, *in vivo* administraction of leupeptin, a reagent that is known to block calpain activity, causes the accumulation of neurofilaments in nerve terminals (Liuzzi, 1990). These observations suggest that calpains can influence neurite elongation by altering the stability of the cytoskeleton.

We tested the hypothesis that calpains mediate Ca^{2+}-induced pruning or regression of neuronal processes using two types of cell-penetrating calpain inhibitors: EST[1], an epoxysuccinyl peptide related to E-64, and MDL 28710, a peptide aldehyde similar to leupetin. These compounds act as substrate analogs and specifically inhibit calpains and other cysteine proteinases (see Wang, 1990; Mehdi, 1991 for reviews). EST (also termed E-64d or loxastatin) is converted by intracellular esterases to its free acid (E-64c), which reacts with the active site thiol of the cysteine residue and irreversibly inactivates the enzyme (Mehdi, 1991). The present study shows that these inhibitors block the Ca^{2+} ionophore A23187-induced regression of neurites in isolated hippocampal pyramidal-like neurons in culture despite rises in intracellular Ca^{2+} levels. A brief summary of these results has been published in abstract form (Song *et al.*, 1990).

MATERIALS AND METHODS

Hippocampal cell cultures

Hippocampi were obtained from 18-old rat fetuses; neuronal cultures were initiated and maintained as described (Mattson *et al.*, 1988b). Briefly, hippocampi were incubated from 15 min in a solution of 2 mg/ml trypsin Ca^{2+}-and Mg^{2+}-free Hank's balanced salt solution buffered with 10 mM HEPES (HBSS; approximately 4~6 hippocampi/ml). The hippocampi were rinsed in HBSS and incubated 5 min in HBSS containing trypsin inhibitor (2 mg/ml). After ringsing in HBSS, cells were dissociated by trituration through the narrowed bore of a fire-polished pasteur pipette and distributed at a density of apprroximately 50 neurons/mm^2 to 35-mm, poly-L-lysine-coated, plastic culture dishes (Corning) containing 1 ml of Eagle's minimum essential medium (MEM; Gibco) buffered with 10 mM sodium bicarbonate and supplemented with 10% fetal bovine serum (Sigma). After 4h of incubation at 37℃ in humidifed 5% CO_2/95% room air atmosphere, the attached cells were washed once with 3 ml MEM and then 1 ml MEM containing 10% Nu-Serum (Collaborative Research) in place of fetal bovine serum, 1 mM pyruvate, and 20 mM KCl was added. Cultures were maintained in this medium at 37℃ in a humidifed 5% CO_2/95% room air atomsphere. All experiments were done on 3-day-old cultures.

Experimental treatments

A23187 was dissolved in ethanol and added to cultures in 5-μl volumes. MDL 28170 (Sigma) and EST were prepared as stocks in ethanol and were diluted in HBSS and were added to cultures in 5- or 10 μl volumes approximately 15 min before the addition of ionophore. Equivalent volumes of vehicle, which were added to control cultures, had no effect on neurite outgrowth or cell survival.

Assessment of neurite ougrowth

Measurements of neurite outgorwth were done

according to previously published methods (Mattson *et al.*, 1988b). Identified neurons were sequentially photographed and tracings of the projected photographic negatives were used to quantify dendritic and axonal elongation rates. Only neurons that had established a characteristic pyramidal-like morphology (Banker and Goslin, 1991) and that were free from contact with other cells were selected from analysis. Statistical comparisions for cell outgrowth were done using Student's paried t-test and values are expressed as the mean±SEM.

Fura-2 analysis of intracellular calcium levels

Methods for measurements of intracellular Ca^{2+} using fura-2 are detailed previously (Mattson *et al.*, 1988b). Briefly, cells were loaded with fura-2/AM (Molecular Probes, Eugene, OR; 2 mM in DMSO diluted to a final incubation concentration of 3 μM) for 30 min, washed, and incubated for 60 min to allow de-esterification. The cultures were viewed on a Zeiss ICM microscope with an RCA S.I.T. camera; the camera output was fed to Quantex (Santa Clara, CA) QA7-210 image processing system where it was converted to a 640× 480 digital image (256 gray levels) and averaged for 540 ms (16 frames). The excitation wavelength was determined by a computer-controlled filter wheel which rotated between 350±10 nm and 380 ±10 nm interference filters. Neurtal density filters (0.12 ND) were inserted in the excitation path to reduce bleaching of the fura-2 and prevent saturation of the camera by the fluorescent emission for the cell body. The fluorescent emission was filtered with a 495 nm long-pass emission filter. Fluorescent images were captured using each exciation filter, the ration (R) of fluorescence intensity (350 nm images)/(380 nm images) was converted to Ca^{2+} concentration using the formula $[Ca]=Kd[(R-Rmin)/(Rmax-R)]$ (Fo/Fs) (Grynkiewicz *et al.*, 1985).

RESULTS

Neurite outgrowth

After three days in culture, embryonic rat hippocampal neurons showed a typical pyramidal cell-like morphology (Banker and Goslin, 1991); each neuron possessed a single and highly elaborate axonal process and several shorter dendritic processes (Fig. 1). The neurites usually showed periods of rapid elongation separated by shorter intervals of outgrowth cessation or neurite regression. As shown previously (Mattson *et al.*, 1988b), dendrites of hippocampal neurons were more sensitive to Ca^{2+} ionophore than were axons. The addition of a low concentration (100 nM) of A23187 to the culture medium induced pruning of dendritic processes without affecting axonal elongation (Figs. 1A, 2A). Incubation of hippocampal neurons with a higher concentration (300 nM) of A23187 resulted in dendritic pruning and a reduction in the rate of axonal elongation (Fig. 2B). In general, 300 nM A23187 produced a greater inhibition in the rate of elongation of dendrites than that of axons (Figs. 2B, 3).

Administration of 5 or 20 μM EST to the culture medium showed dose-dependent protective effects agaist the inhibitory action of 100 nM A23187 on dendritic outgrowth with complete protection at 20 μM EST; there was no effect of EST on axonal outgrowth (Fig. 2A). At the higher concentration (300 nM) of A23187, axonal outgrowth was completely protected at both concentrations of EST, whereas dendritic outgrowth was not protected (Fig. 2B). EST (5 and 20 μM) alone had no significant effect on neurite outgrowth rate (data not shown). The concentrations of calpain inhibitors used in this study did not affect cell survival, as compared to control cultures lacking inhibitor. However, EST was toxic to pyramidal-like neurons at concentrations greater than 100 μM, producing reductions in the number of viable cells comparable to those observed with 1 mM glutamate (Mattson *et al.*, 1988a). In contrast, concentrations of MDL 28170 as high as 300 μM (the highest concentration tested) were not toxic; cell survival after 6h treatment with or without 300 μM MDL 28170 was similar.

MDL 28170 showed the same effect on ionophore-treated hippocampal neurons as EST. At 100 nM A23187, MDL 28170 (20 μM) blocked ionophore-induced suppression of dendritic outgrowth (Fig. 1B). At 300 nM A23187, administraction of either 5 μM or 20 μM MDL 28170 completely blocked ionophore-induced suppression of axonal outgrowth, while dendritic outgrowth was not protected at either concentration

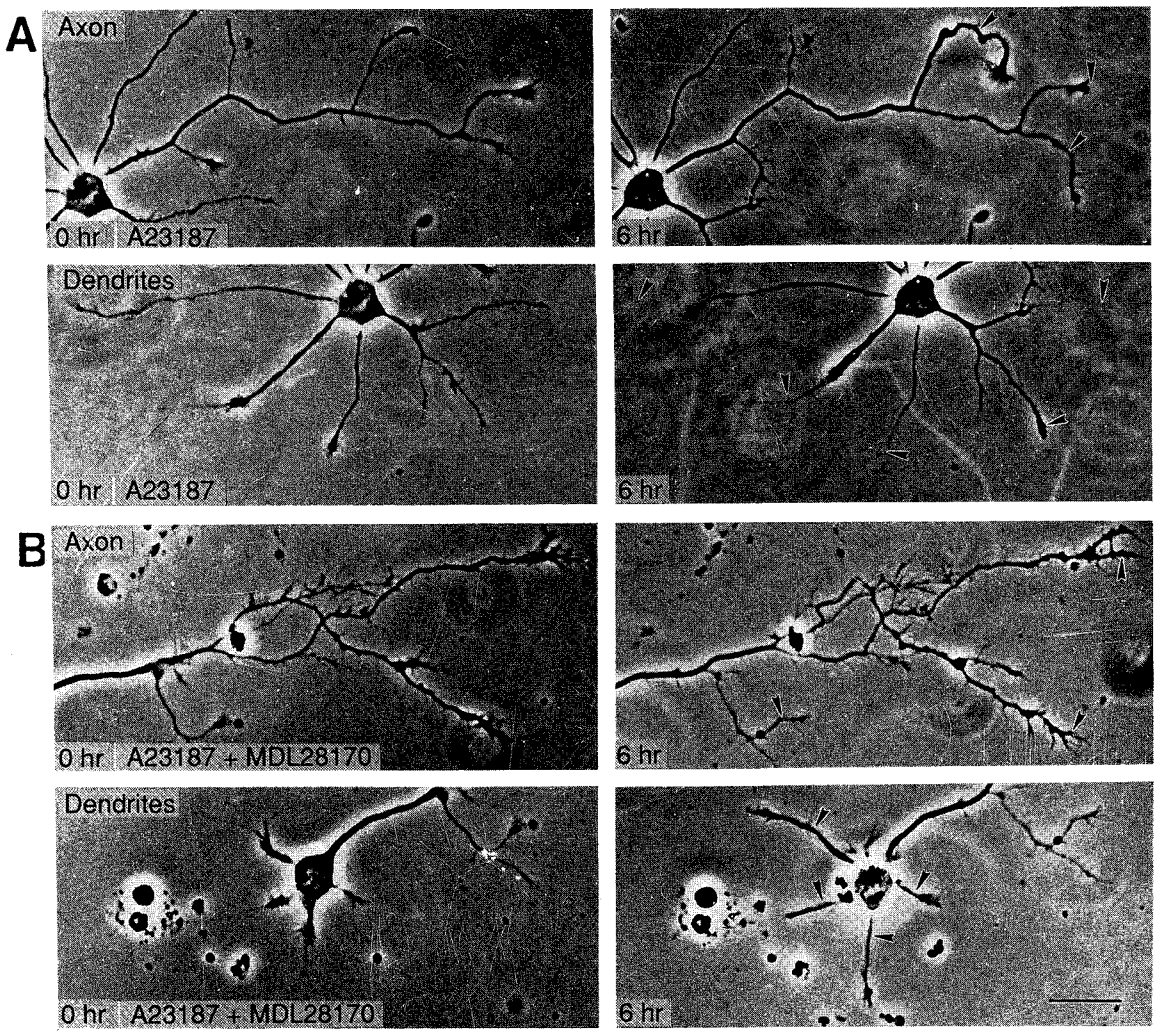

Fig. 1. Effects of A23187 and calpain inhibitor on embryonic rat hippocampal pyramidal cells in culture. (A) Neuron treated with 100 nM A23187 for 0 h (left) and 6 h (right). (B) Neuron treated with 20 μM MDL 28170 and 100 nM A23187 for 0h (left) and 6h(right). Arrowheads in the 6-h micrographs indicate the locations of the termini of axonal and dendritic processes at 0 h. In the presence of ionophore alone outgrowth continued in the axon (A, upper micrographs) while outgrowth slowed or regressed in the dendrites (A, lower micrographs). MDL 28170 blocked the ionophor-induced regression of dendrites (B, lower micrographs) without altering outgrowth of the axon (B, upper micrographs). Scale mark in lower right-hand corner equals 10 μm.

of MDL 28170 (Fig. 3).

Effects of A23187, EST, and MDL 28170 on intracellular Ca^{2+}

While it is assumed that EST and MDL 28170 acted at the level of intracellular calpains, it is possible that these blockers also altered ionophore-induced intracellular Ca^{2+} rises. Measurements of $[Ca^{2+}]_i$ with fura-2 demonstrated that 20 μM EST did not block the 100 nM A23187-induced rise of $[Ca^{2+}]_i$ (Fig. 4). Calpain inhibitor alone had no effect on $[Ca^{2+}]_i$ (data not shown).

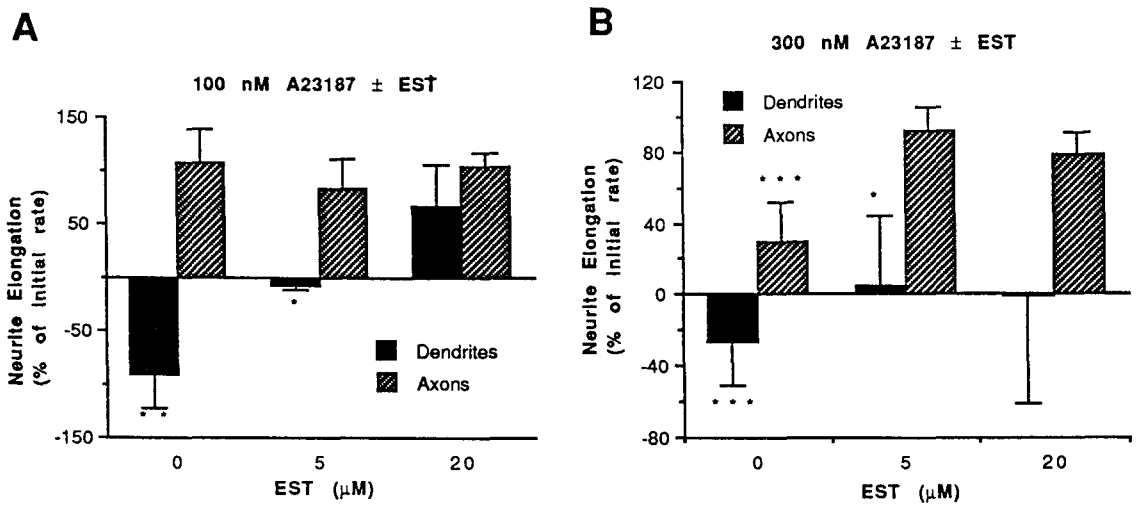

Fig. 2. Effects of EST on A23187-induced changes in neurite outgrowth of rat hippocampal neurons. Rates of axonal and dendritic elongation of neurons grown for three days in culture were determined during a 6-h incubation prior to addition of A23187 and for 6h in the presence of either 100 nM (A) or 300 nM (B) A23187. EST was added 14 min before A23187. Elongation rates during the 6-h exposure to ionophore are expressed as the percent of the initial outgrowth rate measured during the 6-h interval immediately preceeding the addition of ionophore. A value of 100% indicates no change from the initial rate; negative values indicate a reduction in neurite length. Asterisks indicate that neurite outgrowth during the treatment period was significantly different from the initial rate at $p<0.01^{***}$, $p<0.02^{**}$, or $p<0.05^{*}$. (A) A low concentration (100 nM) of A23187 induced a regression of the dendrites, which was blocked by 20 μM EST. Data is presented as the mean and SEM of determinations made on 17 to 23 neurons (3 or 4 separate cultures) for each treatment. The initial elongation rates of axons and dendrites were 4.9 ± 0.6 and 1.9 ± 0.6 μm/h, respectively. (B) A higher concentration (300 nM) of A23187 slowed axonal elongation, as well as induced dendritic regression. Both concentrations of EST protected the axons, but not the dendrites, from the ionophore. The apparent protection of dendritic outgrowth at 20 μM EST probably resulted from the large SEM in that sample of cells. Data is presented as the mean and SEM of determinations made on 22 to 40 neurons (4 or 5 separate cultures) for each treatment. The initial elongation rates of axons and dendrites were 4.6 ± 0.6 and 1.9 ± 0.7 μm/h, respectively.

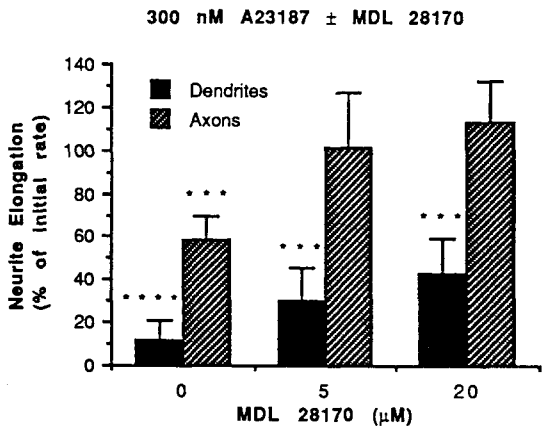

Fig. 3. Effects of MDL 28170 on A23187-induced changes in neurite ourgrowth of rat hippocampal neurons. Rates of axonal and dendritic neurite elongation of neurons grown for three days in culture were determined as in fig. 2 and expressed as the percent of the initial rate; a value of 100% indicates no change from the initial rate. MDL 28170 was added 14 min before the addition of 300 nM A23187. Asterisks indicate that neurite outgrowth during the treatment period was significantly different from the initial rate at $p<0.001^{****}$ or $p<0.01^{***}$. Both concentrations of MDL 28710 protected the axons, but not the dendrites, from the ionophore. Data is preseted as the mean and SEM of determinations made on 19 to 64 neurons (4 or 5 separate cultures) for each treatment. The initial elongation rates of axons and dendrites were 6.2 ± 0.7 and 2.4 ± 0.4 μm/h, respectively.

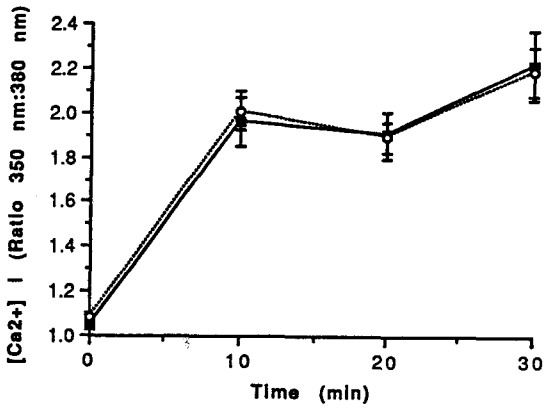

Fig. 4. Effect of EST on A23187-induced rise in intracellular Ca^{2+}. A23187 (100 nM), which was added to the culture medium at 0 min, caused identical increases in intracellular Ca^{2+} in hippocampal neurons incubated with ionophore alone (□) or with ionophore and 20 μM EST (●). Values represent the mean and SEM (n= 28, 29, 23, and 19 at 0, 10, 20, and 30 min, respectively of 2 separate cultures treated with A23187 alone; n=88, 36,34, and 28 at 0, 10, 20, and 30 min, respectively of 3 separate cultures treated with EST and A23187).

DISCUSSION

A23187-induced neurtie pruning in rat hippocampal neurons appears to involve the hydrolysis of cytoskeletal elements by calpains. Numerous studies have shown that external application of calpain inhibitors, such as calpeptin, EST, and MDL 28170, prevent calpain activation in a variety of cell types (Wang, 1990; Mehdi, 1990). For example, in erythrocytes and platelets, EST and MDL 28170 penetrate the cell membrane and inhibit the calpain-mediated degradation of cytoskeletal proteins, such as spectrin, actin-binding protein, and talin (Mehdi *et al.*, 1988; Fox *et al.*, 1991). In the rat hippocampal neurons, EST and MDL 28170 inhibited calpains without affecting the ionophore-induced rise in intracellular Ca^{2+}, since measurements of intracellular Ca^{2+} with fura-2 showed that the increase in intracellular Ca^{2+} was identical between hippocampal neurons treated with A23187 alone or with A23187 and EST (Fig. 4).

The differential sensitivity of dendrites and axons to A23187 may be due to differences in calcium homeostasis, calpain distribution, and/or the cytoskeleton. Ca^{2+}-buffering or Ca^{2+}-extruding capability may be greater in axons than in dendrites and thus the higher concentration of ionophore is required to attain a similar increase in intracellular Ca^{2+} in axons as in dendrites incubated with 100 nM A23187. Glutamate and other excitatory amino acids produce higher sustained Ca^{2+} levels in dendrites than in axons of hippocampal neurons (Connor *et al.*, 1988). Differences in the intracellular distribution of calpain and/or calpastatin may also contribute to this differential sensitivity. In fact, in hippocampal cells fixed *in situ*, calpain I immunoreactivity is preferentially localized in the dendrites (Perlmutter *et al.*, 1990; Fukuda *et al.*, 1990). Lastly, cytoskeletal proteins of the dendrites may be more vulnerable to calpains than those of the axons. MAP2, which is readily hydrolyzed by calpains (Billger *et al.*, 1988) and is restricted to the dendrites (Morales and Fifkova, 1989), may play an important role in stabilizing the cytoskeleton, and thus make the dendritic cytoskeleton more susceptible to Ca^{2+}-dependent proteolysis. Conversely, the phosphorylated form of tau protein, which is restricted to the axons, is less sensitive to proteolysis by calpain (Litersky and Johnson, 1992).

The calpain inhibitors EST and MDL 28170 block the A23187-induced suppression of neurite outgrowth. This protection of neurites against A23187, however, was only effective at low concentrations of ionophore (100 and 300 nM for dendritic and axonal processes, respectively). These concentrations induce increases in intracellular Ca^{2+} (see Kater and Mills, 1991 for review). EST or MDL 28170, however, were not effective at higher concentrations (1 μM) of A23187 (data not shown), indicating that larger influxes of Ca^{2+} induce changes in cellular metabolism that are mediated by Ca^{2+}-dependent processes in which calpains are not involved. This suggests that excitotoxitciy and other neurodegenerative processes associated with large increases in intracellular Ca^{2+} involves more than a simple activation of calpains (Siman *et al.*, 1989). In rat cerebellar

granule neurons, for example, leupeptin prevents calpain activation (as determined by degradation of spectrin), but has no effect on glutamate neurotoxicity (Manev *et al.*, 1991). Other Ca^{2+}-dependent hydrolases, such as phospholipase C and A_2 and Ca^{2+}-activated endonuclease(s), may be involved in Ca^{2+}-mediated cell death (see Nicotera *et al.*, 1992 for review). However, subtoxic concentrations (50 μM) of glutamate mimic the effects of low concentrations (50~100 nM) of A23187 on neurite outgrowth of hippocampal cells (Mattson *et al.*, 1988b). Since glutamate also raises intracellular Ca^{2+} (Lipton and Kater, 1989; Connor *et al.*, 1988; Mattson *et al.*, 1988b), the results of this study suggest that calpains mediate the modification of neuronal form by neurotransmitters during normal development and plasticity (Mattson *et al.*, 1988c).

In the absence of A23187, EST and MDL 28170 had no effect on neurite outgrowth of rat hippocampal pyramidal neurons. This suggests that, under the culture conditions employed, calpains are maximally suppressed and/or down-regulated to permit elongation of the neurites. The results of this study show that the calpains must first be activated by an influx of Ca^{2+} before any effect of calpain inhibitor is observed. It is possible that culture conditions reduced the calpain activity in the hippocampal neurons, either by down-regulating calpain or up-regulating calpastatin. In PC12 cells, for example, nerve growth factor-induced neuritogenesis is correlated with a decrease in calpain activity caused by increased levels of calpastatin (Oshima *et al.*, 1989). It therefore appears that calpain activity can have a profound effect on neuroarchitecture, and that changes in expression of calpains and/or their endogenous regulators determine whether neurites grow or regress.

A role for calpains in neurite outgrowth rests on the findings that cytoskeletal proteins are preferred substrates for these enzymes (Shoeman and Traub, 1990) and that calpain inhibitors stimulate neurtie outgrowth (Saito and Kawashima, 1989; Shea *et al.*, 1991, 1992) and induce accumulation of neurofilaments in nerve endings (Liuzzi, 1990). However, since none of compounds available specifically inhibit calpains, other cysteine proteinases cannot be excluded. E-64 analogs and MDL 28170 rapidly inactivated papain and cathepsins B, H, and L, as well as calpain (Barrett *et al.*, 1982; Wang, 1990; Mehdi, 1991). In addition, incubation of [^{14}C]-EST with epidermoid A431 cells in culture labels endogenous proteins other than calpains (Shoji-Kasai *et al.*, 1988), which raises the possibility that EST affects other biochemical pathways. However, we have shown that both EST and MDL 28710 suppress a Ca^{2+}-dependent process. The calpains are the only intracellular cysteine proteinases that are activated by Ca^{2+} (see Bond and Butler, 1987 for review). Three of four Ca^{2+}-dependent metalloproteases have been identified in human and rat brain, respectively; these enzymes, however, are not inhibited by leupeptin or sulfhydryl reagents and thus are not related to calpain (Nelson and Siman, 1989; Backstrom *et al.*, 1992). The results of this study strongly suggest that calpains mediate the ionophore-induced regression in hippocampal cells, although the disruption of other Ca^{2+}-dependent enzymes by calpain inhibitors remains a remote possibility.

Although it is not known which calpain isozyme mediates A23187-induced neurite pruning, the avalilable evidence suggests that calpain I is involved. In the rat hippocampus, immunocytochemical studies have shown that calpain I is the predominant form in pyramidal neurons; calpain II is nearly absent (Fukuda *et al.*, 1990). The low concentrations of ionophore required to induced neurtie regression and the measurements of intracellular Ca^{2+} with fura-2 suggest that Ca^{2+} concentrations never rise high enough to activate calpain II. It remains possible that association of calpain II with phospholipids of the cell membrane, which lowers the Ca^{2+} requriement of the enzyme (Coolican and Hathaway, 1984), combined with highly localizd influxes of Ca^{2+}, may be adequate to activate calpain II. In serum-deprived mouse neuroblastoma cells, calpain I appears to involved, since N-acetyl-leucyl-leucyl-norleucinal, which preferentially inhibits calpain I, stimulates neurite outgrowth, whereas N-acetyl-leucyl-leucyl-methional, which preferentially inhibits calpain II, is without effect (Shea *et al.*, 1991).

There are multiple parallel signal transduction pathways, both Ca^{2+}-dependent and Ca^{2+}-independent, that regulate neuronal morphology. Ca^{2+} may direct elongation and growth cone motility by regulating microtubule polymerization and microfilament-based motility, respectively (Lank-

ford and Letourneau, 1989). Neuroarchitecture may also be affected by the activity of Ca^{2+}-dependent enzymes other than calpains, such as protein kinase C and calmodulin-dependent protein kinases, and cAMP-dependent protein kinase on cytoskeletal proteins (Mattson *et al.*, 1988b; Polak *et al.*, 1991). Phosphorylation of MAP2 by either cAMP-or calmodulin-dependent protein kinase reduces the ability of MAP2 to polymerize microtubules (Olmsted, 1986). Differential expression of MAP2 and tau protein variants (Nunez, 1988), as well as phosphorylation of neurofilaments (Clark and Lee, 1991) and tau protein (Litersky and Johnson, 1992), may also influence cytoskeletal organization. We have shown that under specific conditions in which hippocampal neurons experience an influx of Ca^{2+}, calpains mediate the suppression of neurite elongation, probably by degrading cytoskeletal proteins at the growth cone.

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=국문초록=

해마추상체 신경세포에서 칼슘에 의한 신경섬유 성장억제에 대한 칼파인 억제제의 영향

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칼슘이온은 신경섬유 성장의 중요한 조절인자이나 그 정확한 작용기전은 불명확하다. 세포골격 단백은 *in vivo* 및 *in vitro*에서 칼슘의존성 단백분해효소(칼파인)에 의해 신속히 분해되므로, 칼슘이온에 의한 신경섬유의 퇴행에 있어서 칼파인의 관련성을 추구하기위하여, 배양된 해마신경세포에서 칼슘이온 ionophore인 A23187에 의한 신경섬유의 성장억제가 칼파인의 억제제인 EST 및 MDL 28170에 의해 차단되는지를 조사하였다. A23187은 100 nM의 농도에서 축삭에는 영향이 없이 수상돌기의 퇴행을 유발하였으나, 300 nM의 농도에서는 축삭의 성장을 억제하였다. EST(5 혹은 20 uM) 및 MDL 28170(20 uM)은 100 nM A23187의 수상돌기에 대한 작용과 300 nM A23187의 축삭에 대한 작용을 효과적으로 차단하였다. EST는 A23187에의한 세포내 칼슘이온의 증가를 차단하지 못하였다. 이상의 결과는 해마추상체세포에서 칼슘에 의한 신경섬유의 퇴행이 칼파인에 의해 매개됨을 시사한다.